МИКРОБИОЛОГИЯ

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A Translation of

# MICROBIOLOGY

*(Mikrobiologiya)*

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# MICROBIOLOGY

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ERRATA

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On p. 627, in Table 2, the figures for "Average" at the bottom of the first three columns of the right-hand half of the table should read 5.2, 4.0, and 4.2; the figure at the bottom of the fourth column should be deleted.

On p. 628, the figure 8.4 in the last column of Table 5 should be changed to 3.4.

# VITAL SECRETION OF REDUCING SUBSTANCES IN MICROBIAL CULTURES

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It is well known that during the growth of any microorganism a drop in the oxidation–reduction potential can be detected in the culture.

The reasons for this drop are not clear. The lowering of the oxidation–reduction potential detected by platinum electrodes or by $rH_2$ indicators may be due to several causes: the consumption by the microorganism of the oxygen dissolved in the medium; the enrichment of the medium by hydrogen, which is frequently a metabolic product of microorganisms; the release into the medium of various vital products, such as hydrogen sulfide, aldehydes, formic acid, etc., which have reducing properties.

A drop in the potential occurs in any growing culture of aerobes or anaerobes under the condition that the medium is not subjected to vigorous aeration. However, even during cultivation on shakers a lowering of the potential can frequently be observed in cultures of aerobic microorganisms, which suggests a high rate of production of reducing substances.

It is unlikely that the drop in potential is incidental. It can be assumed that this is an adaptation ensuring better growth of the culture. The basis for such an assumption is the fact that the addition to the culture of reducing substances of the most varied nature–sodium hydrosulfite or ascorbic acid–shortens the lag phase and accelerates the reproduction of aerobes. Reducing substances apparently protect the cell from excessive oxidation (Rabotnova, 1957; Rabotnova and Mineeva, 1959).

The aim of the present work is to study the secretion of reducing substances by microorganisms during the period of rapid multiplication.

Reducing substances in microbial cultures. First of all, the very fact of the accumulation of reducing agents in cultures during growth of microorganisms should be looked into more carefully, because the simple removal of dissolved oxygen during the course of respiration can also cause a lowering of the potential as measured by electrodes or indicators. For this purpose, $rH_2$ measurements were made in cultures of microorganisms on different media and on the same sterile media, both in contact with air and when placed under anaerobic conditions. In the latter case, special glass test tubes with sealed-in platinum electrodes were used in which anaerobic conditions could be created by removing oxygen with such chemical absorbents as pyrogallol or hydrosulfite, and Eh measurements could simultaneously be made under sterile conditions (details in Rabotonova, 1957).

The experiment showed (Table 1) that the removal of dissolved oxygen from such a complex medium as meat-peptone broth leads to a sharp reduction in $rH_2$ from 25 to 16.2. This is evidence of the presence in the medium of reducing substances acting similarly to reducing sugars (Wurmser, 1935), the reducing capacity of which becomes apparent after the removal of oxygen; the $rH_2$ of simple defined media consisting of the usual complement of mineral salts and such substances as citrate, mannitol, and glycerol, which do not have reducing properties, differs insignificantly–only by two units–under aerobic and anaerobic conditions. During the growth of a number of cultures, the $rH_2$ decreases even more than in the same medium under anaerobic conditions. This

TABLE 1

The $rH_2$ of Sterile Media under Aerobic and Anaerobic Conditions and during the Growth of Microorganisms

| Medium | Sterile medium | | During growth of cultures | |
|---|---|---|---|---|
| | aerobically | anaerobically | culture | minimal $rH_2$ value |
| Meat-peptone broth | 25 | 16.2 | Bacillus subtilis | 14.7 |
| | | | Pseudomonas fluorescens | 12.4 |
| | | | Pseudomonas pyocyaneus | 13.0 |
| | 20 | 16.5 | Escherichia coli | 13.0 |
| Defined medium with potassium citrate and ammonia | 26 | 23.9 | Pseudomonas pyocyaneus | 17.8 |
| | | | Pseudomonas boreopolis | 20.0 |
| | | | Bacillus mesentericus | 20.0 |
| The same with mannitol and $KNO_3$ | 25,9 | 24.0 | Pseudomonas pyocyaneus | 19.3 |
| The same with glycerol and $KNO_3$ | 25 | 23 | Actinomyces venezuelae | 10.1 |

occurs on both meat-peptone broth and on defined media. Consequently, reducing substances are produced during growth of the cultures.

TABLE 2

Changes in $rH_2$ in a Culture and Culture Centrifugate of Torula utilis without Access of Air during the Course of its Growth

| Exp. No. | Age of culture, hr | No. of cells in culture, millions / ml | In centrifugate without access of air | | |
|---|---|---|---|---|---|
| | | | pH | Eh, mv | $rH_2$ |
| 1 | 0 | 1.20 | 5.65 | +235 | 18.5 |
| | 3 | 1.37 | 5.65 | +175 | 17.3 |
| | 7 | 2.0 | 5.65 | +210 | 18.3 |
| | 12 | 3.9 | 5.65 | +170 | 17.2 |
| | 24 | 5.2 | 5.70 | +135 | 16.0 |
| | 34 | 14.8 | 5.85 | +50 | 13.4 |
| | 37 | 80.8 | 7.50 | —110 | 11.3 |
| | 46 | 120 | 8.4 | —150 | 11.8 |
| | 70 | 123 | 8.4 | —10 | 16.5 |
| 2 | 0 | 104 | 5.5 | +300 | 21 |
| | 6 | 161 | 6.1 | +50 | 13.9 |
| | 8 | 241 | 7.3 | +50 | 16.3 |
| | 10 | 260 | 7.8 | +50 | 17.3 |
| | 31 | 258 | 8.5 | —10 | 16.7 |

Reducing substances in the centrifugate of a Torula utilis culture. The question arises: Can these substances be detected in cell-free culture fluid ? In order to answer this question, the microorganisms had to be grown on a medium containing no reducing substances in its composition. Sugar-containing media are unsuitable for this purpose, since in slightly alkaline medium reducing sugars produce a low Eh. Only those microorganisms which do not require amino acids and vitamins grow in simple synthetic media containing the salts of organic acids or glycerol as the sole carbon source. Of the many cultures tested on simple media, actinomycetes and Torula utilis gave the best growth.

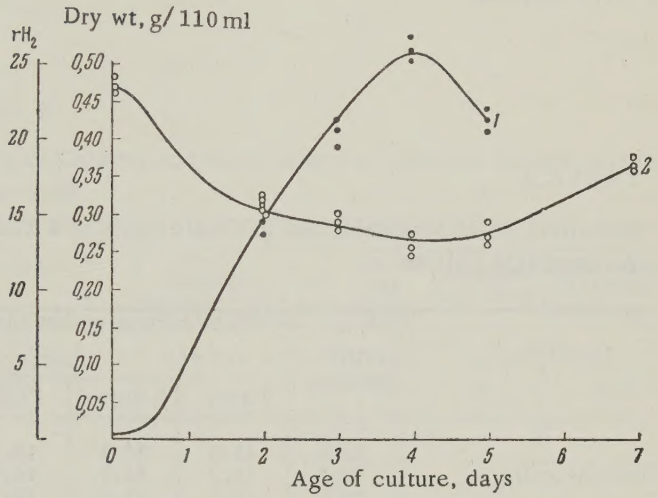
Torula utilis was grown on medium of the following compositon (in g): potassium citrate-5; ammonium citrate-5; $KH_2PO_4$-1; $MgSO_4$-0.5; $FeSO_4$-0.03; $ZnSO_4$--0.05; tap water-1 liter; pH 5-5.5. As the culture grew on a shaker rotating at 200 rpm, samples were taken, the cells were removed by centrifugation, and the pH and Eh of the centrifugate were determined. When the centrifugate was in contact with air, its potential was always high, as was the case with sterile medium. We then placed the centrifugate under anaerobic conditions and measured the Eh without access of air, using the small tubes mentioned before. Thymol was added to the centrifugate in an amount which would ensure the complete absence of the vital activity of any microorganisms. This was necessary because anaerobic conditions did not become established immediately, but over a period of several hours at 35 C. Samples were collected at the time of the most rapid multiplication before the number of cells reached its maximum and before autolytic manifestations began to appear in the culture, although no autolyzing cells were noted microscopically even in a 7-day culture of T. utilis.

Data are presented in Table 2 which were obtained with cultures for which small (1) and massive (2) inocula were used.

TABLE 3

Reversible Oxidation of Reducing Substances in a Torula utilis Centrifugate

| Expt. No. | Eh in anaerobic conditions, mv | Eh after bubbling air for 1 hr, mv | Eh after again placing under anaerobic conditions, mv | Eh after boiling under anaerobic conditions, mv |
|---|---|---|---|---|
| 1 | −35 | +290 | −30 | +300 |
| 2 | −30 | +300 | −25 | +270 |
| 3 | +50 | +290 | +30 | +280 |




Growth of Actinomyces venezuelae and $rH_2$ values in culture centrifugates without access of air. 1) Dry weight of mycelium; 2) $rH_2$ of centrifugate without access of air.

It can be seen from Table 3 that the cells released some substances into the medium which, in the absence of autolysis, produced a low oxidation-reduction potential. The low potential was found only under anaerobic conditions. The same centrifugate could be repeatedly aerated and again placed under anaerobic conditions, and the Eh in it would change markedly with this treatment. After boiling for 20 min the capacity for reversible oxidation-reduction decreased, which speaks for the enzymatic nature of this process.

No substances containing the sulfhydryl group (sodium nitroprusside reaction), ascorbic acid, or substances reducing Fehling's solution were found in the centrifugate.

Reducing substances in the centrifugate of an Actinomyces venezuelae culture. Similar experiments were set up with A. venezuelae cultured on a medium of the following composition (in g): glycerol-20; $K_2HPO_4$-0.5; $MgSO_4$-0.5; $KNO_3$-3; NaCl-0.5; $FeSO_4$-traces; tap water-1 liter; pH 7.3-7.4.

In the figure, the $rh_2$ values of the centrifugate under anaerobic conditions are plotted on a curve of the changes in dry weight of the mycelium during the course of growth of the culture. The greatest reducing capacity was found during the period of the most rapid accumulation of biomass. Particular attention should be paid to the

2- and 3-day cultures. In this period, there was still no autolysis. The mycelium stained evenly with gentian violet without fragmentation, which is the first stage of autolysis. Unused nitrate nitrogen was still present in the medium.

The reversibility of the oxidation-reduction in the centrifugate is seen from the data presented in Table 4.

After boiling for 20 min, the reversible oxidation-reduction ceased.

The reducing capacity of centrifugates under anaerobic conditions can be detected not only with platinum electrodes, but with $rH_2$ indicators as well. In the centrifugate without access of air, thionine, 2,6-dichlorophenol-indophenol, and tetrazole were reduced.

Protein was detected qualitatively in the centrifugate by the Fraenkel-Conrat method (microbiuret reaction). When the total nitrogen, nitrate nitrogen, and nitrogen after precipitation of protein according to Barnshtein were determined quantitatively, it was found that the culture released about 1 mg of nitrogen, including 0.35-0.70 mg of protein nitrogen, per 100 ml of medium.

TABLE 4

Reversible Oxidation–Reduction in Centrifugates of a Young A. venezuela Culture

| Conditions | $rH_2$ of sterile medium | $rH_2$ of culture centrifugate at | | |
|---|---|---|---|---|
| | | 2 days | 3 days | 4 days |
| Anaerobically | 22.9 | 15.0 | 14.4 | 13.1 |
| | 23.0 | 15.3 | 14.7 | 14.6 |
| | 23.8 | 15.7 | 15.3 | 12.7 |
| After bubbling of air | 24.2 | 25.3 | 27.1 | 27.5 |
| | 25.6 | 26.6 | 28.3 | 28.6 |
| | 25.8 | 25.4 | 24.3 | 26.6 |
| Again anaerobic | 23.2 | 16.2 | 15.8 | 14.9 |
| | 23.4 | 17.7 | 16.3 | 16.9 |
| | 23.9 | 15.2 | 17.0 | 15.0 |
| After second bubbling | 24.6 | 25.8 | 27.6 | 27.8 |
| | 25.9 | 26.3 | 28.4 | 28.8 |
| | 25.8 | 25.3 | 24.5 | 27.0 |
| After boiling, anaerobic | — | 24.4 | 21.6 | 22.8 |
| | | 21.2 | 23.5 | 24.7 |

Reducing substances in centrifugates and cultures of some bacteria. We attempted to detect reducing substances during the growth of two strains of Pseudomonas pyocyaneus, Pseudomonas boreopolis, Bacillus megaterium, and Bacillus mesentericus on simple defined media with potassium citrate or mannitol, the salts of pyruvic or fumaric acids, and nitrate or ammonium nitrogen. All of the bacteria tested grew rather poorly on these media. The maximal number of cells did not exceed 40-70 million/ml, while on meat-peptone broth under the same conditions of good aeration on shakers, as many as several billion cells grew per 1 ml. The Eh was measured in the cultures at the time of the greatest rate of multiplication. Centrifugates of cultures without access of air, suspension of centrifuged cells, as well as centrifugates of old, autolyzing cultures without access of air were tested (Table 5).

TABLE 5

The $rH_2$ of the Centrifugates and in the Centrifuged Sediment of Bacterial Cultures on Media with Potassium Citrate and Ammonium Citrate

| Culture | $rH_2$ | | | | |
|---|---|---|---|---|---|
| | in sterile medium, anaerobically | cultures | centrifugate of sediment, aerobically | centrifugate of young culture, anaerobically | centrifugate of autolyzed culture, anaerobically |
| P. pyocyaneus 1 | 23.9 | 17.8 | 19.1 | 24.3 | 19.5 |
| P. pyocyaneus 2 | 23.9 | 18.0 | 19.8 | 23.8 | 18.0 |
| P. boreopolis | 23.9 | 20.0 | 19.5 | 23.8 | 17.3 |
| B. megaterium | 23.9 | 21.3 | 19.4 | 24.0 | 18.1 |
| B. mesentericus | 23.9 | 20.0 | 19.3 | 25.2 | 19.1 |

TABLE 6

The $rH_2$ of Cultures and Centrifugates of Bacterial Cultures on Meat-Peptone Broth

| Culture | $rH_2$ | | | | | |
|---|---|---|---|---|---|---|
| | sterile medium | | cultures | bacterial sediment | supernatant fluid | supernatant fluid after bubbling air |
| | aerobically | anaerobically | | | | |
| P. pyocyaneus | 23 | 18.8 | 14.8 | 14.0 | 19.9 | 21.2 |
| B. subtilis | — | — | 15.2 | 15.0 | 20.3 | 20.3 |
| " " | 23 | 16.8 | 16.3 | 16.5 | 20.0 | 20.7 |
| " " | — | — | 15.2 | — | — | — |
| " " | — | — | 14.7 | — | — | — |

In distinction from Torula utilis and A. venezuelae, no reducing substances were found in centrifugates of young bacterial cultures before the onset of autolysis when measurements were made under anaerobic conditions. In the centrifuged sediment, considerable reducing capacity was found even with the access of air. Reducing capacity was also found in centrifugates of autolyzing cultures. Reducing substances are apparently released during the breakdown of cells. Young cells evidently produce reducing substances capable of lowering the $rH_2$ without access of air, but they are not released into the medium in a young culture, but are apparently associated with the cell surface.

As has already been pointed out, meat-peptone broth contains substances which sharply lower the Eh of sterile medium under anaerobic conditions. Therefore, the release of reducing substances may be masked by the activity of substances already present in the medium. We attempted to determine whether it is possible to detect autooxidizing reducing substances in culture centrifugates immediately after the separation of the cells from the fluid. The bacterial cultures were grown on meat-peptone broth in centrifuge tubes. At the moment of maximal reduction of $rH_2$, the cultures were centrifuged in the same tubes. Electrodes were placed in the bacterial sediment and in the supernatant fluid, and measurements were made immediately. The results are presented in Table 6.

The data presented show that reducing substances are found directly on living cells, and either do not pass into the fluid or are oxidized by the oxygen of the air immediately after separation from the cells.

## DISCUSSION OF RESULTS

In microorganisms, their minute size explains,not only quantitative, but also qualitative,differences from higher organisms. Microorganisms can hardly protect the contents of their cells from the environment as completely as do higher plants and animals. The connection of microorganisms with the environment is undoubtedly much closer than in higher organisms. They are obliged not only to protect themselves from the environment, but to actively adapt to this environment as well and to make it more favorable for themselves, especially during the period of intensive reproduction of the culture.

The nature of aerobic microorganisms is contradictory. On the one hand, they require oxygen from the air in order to carry out energy-yielding processes. On the other hand, extreme aeration is unfavorable for them, since it can put out of commission such enzyme systems as coenzyme A, for example, which is active only in the reduced state. Aerobes can apparently regulate the access of oxygen to the cells in some manner. Some of the processes occurring inside the cell in higher organisms may possibly be carried out in the external medium or on the cell surface in microorganisms. It is easily assumed that the first stages of transfer of the hydrogen of the oxidizable substrate to oxygen occur inside the cell in microorganisms. However, the direct transfer of hydrogen to oxygen, connected with the functioning of carriers with a high oxidation–reduction potential, such as cytochrome oxidase or substances similar to it, may occur on the cell surface or even in the medium.

In all probability, the reversible oxidation–reduction systems which we found in the centrifugates of T. utilis and A. venezuelae are terminal oxidases in the oxidative processes of these obligate aerobes. They accept oxygen without allowing it to penetrate into the cell, serving as a unique protective barrier against excessive oxidation. During oxygen deficiency, they are in the reduced state, making for the low oxidation–reduction potential which is so characteristic for all microbial cultures in the state of active multiplication and deficiency of oxygen dissolved in the medium.

## SUMMARY

1. The decrease in Eh in the cultures of some bacteria, yeast and actinomycetes is due not only to absorption by the culture of oxygen dissolved in the medium but also the formation of reducing substances.

2. Torula utilis and Actinomyces venezuelae release reducing substances into the medium. These are thermolabile reversible oxidation–reduction systems.

3. In Pseudomonas pyocyaneus, Pseudomonas boreopolis, Bacillus megaterium and Bacillus mesentericus, no reducing substances have been detected in the medium. Only living cells exhibit a high reducing capacity.

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**All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.**

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# A NEW SPECIES OF NITROGEN-FIXING MYCOBACTERIUM AND ITS PHYSIOLOGICAL PROPERTIES

M. V. Fedorov and T. A. Kalininskaya

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In our preceding communication (Fedorov and Kalininskaya, 1959), data were presented on the presence in the composition of the microflora of turfpodzol soils of oligonitrophilic bacteria which fix considerable amounts of molecular nitrogen under conditions of mixed culture with other bacteria. In specific cases, the efficiency of nitrogen fixation reached 10-15 mg per 1 g of glucose consumed, 10-11 mg per 1 g of ethyl alcohol consumed, and 5-6 mg per 1 g of acetic acid consumed. Mixed cultures of this type were isolated by us from the soils of the field experimental station of the Timiryazev Academy of Agriculture (9P and 15P) and from the rhizosphere of oats (9K, 15K, and 25K) growing in these soils. The rather high nitrogen fixing activity of these cultures prompted us to study their species composition in order to establish precisely which bacteria participate in this process. However, we encountered rather considerable difficulties in the initial stages of the investigation. The principal one of these consisted in the fact that not a single one of the bacteria isolated from these mixtures as pure cultures had the ability to fix molecular nitrogen under pure culture conditions. This circumstance greatly complicated our problem; nevertheless, we gradually succeeded in determining the reason for such unusual behavior by these bacteria. It proved to be associated with their physiological peculiarities, particularly with the inability of the nitrogen-fixing bacterium to assimilate glucose as a carbon source and a markedly pronounced requirement for supplementary growth factors.

These peculiarities were established in the following manner: Pseudomonas radiobacter, Flavobacterium fulvum and mycobacterium (strain 301) were isolated in pure cultures from mixed culture 25K (b). By means of consecutive inoculations of each of these bacteria into nitrogen-free nutrient medium with glucose, it was quite accurately established that both in pure culture and in combination with one another, the first two bacteria do not assimilate molecular nitrogen in either case. On this basis, the nitrogen fixing activity of the original mixed culture had to be regarded as associated with mycobacterium 301. However, not only the isolation of this bacterium into pure culture, but even its detection in the mixed culture, was extremely difficult due to the fact that, both on nitrogen-free nutrient media with glucose and on the same media to which mineral nitrogen had been added, it either did not grow at all or gave extremely slow and poor growth, sometimes detectable only upon examination of the Petri dishes under the microscope. This situation was explained by the fact that this bacterium does not utilize glucose as a carbon source during growth in pure culture, although in mixed cultures this carbohydrate is the best carbon source. On the other hand, when this bacterium is cultured on meat-peptone agar, its growth is greatly inhibited by the organisms accompanying it—P. radiobacter and F. fulvum—which also makes its isolation difficult. It was all of these peculiarities of mycobacterium 301, which became apparent only subsequently, which made its isolation in pure culture difficult.

When it was established by means of various experiments that P. radiobacter and Flavobact. fulvum cannot fix atmospheric nitrogen in the absence of mycobacterium 301, it became obvious that it is this bacterium which performs the given function. However, in order to ascertain that it is indeed the active fixer of molecular nitrogen in mixed cultures, additional experiments were set up in which a number of other soil microorganisms not having the ability to assimilate atmospheric nitrogen were inoculated, together with this bacterium.

The results of these experiments are given in Table 1.

TABLE 1

Fixation of Molecular Nitrogen by Mycobacterium 301 in Mixed Cultures (average data of three repetitions)

| Species composition of bacterial cultures | Nutrient medium | Glucose utilized, % | Atmospheric nitrogen fixed per 30 ml of medium, mg | Efficiency of nitrogen fixation, mg/g of glucose |
|---|---|---|---|---|
| Mycobacterium 301 | Without nitrogen + 0.39 mg of yeast autolyzate nitrogen | 0.0 | 0.0 | 0.0 |
| | | 0.0 | 0.0 | 0.0 |
| Mycobacterium 301 + + P. radiobacter | The same | 100.0 | 3.42 | 12.22 |
| | | 100.0 | 4.20 | 14.95 |
| Mycobacterium 301 + + F. fulvum | The same | 10.0 | 0.0 | 0.0 |
| | | 100.0 | 2.32 | 8.29 |
| Mycobacterium 301 + + P. fluorescens | The same | 100.0 | 3.06 | 10.9 |
| | | 100.0 | 4.08 | 14.59 |
| Mycobacterium 301 + + B. coli | The same | 100.0 | 2.90 | 10.36 |
| | | 100.0 | 2.22 | 7.71 |
| Mycobacterium 301 + + Chromobacterium prodigiosum | The same | 100.0 | 3.78 | 13.51 |
| | | 100.0 | 1.60 | 5.73 |

From these data, it is easily established that the fixation of atmospheric nitrogen was found only in those experimental variants which contained mycobacterium 301. On the other hand, the pure culture of this bacterium on glucose could not grow and, by virtue of this, could not fix atmospheric nitrogen. Since, in this experiment, nearly the same nitrogen fixing activity was observed in mixtures of this bacterium with various other bacteria, it must be concluded that its "symbiosis" with them is not of a specific nature. Apparently, all bacteria used in the experiments form incomplete oxidation products (ethyl alcohol, acetic and lactic acids) during the breakdown of glucose which serve as available carbon sources for this bacterium. In addition, they may also supply it with supplementary growth factors. The latter possibility became evident following experiments in which this bacterium was grown on nitrogen-free media containing carbon sources available to it (with ethyl alcohol) both in pure culture and in a mixture with other bacteria. In pure culture on nitrogen-free medium, it grew comparatively poorly on ethyl alcohol as well and did not fix atmospheric nitrogen. However, when yeast autolyzate containing 0.8 mg of bound nitrogen was added to the medium, nitrogen fixation was 4.71 mg of nitrogen per 1 g of alcohol consumed. This indicates that supplementary growth factors present in yeast autolyzate are of considerable importance for the growth of this bacterium and provide more favorable conditions for nitrogen fixation. Approximately the same efficiency of this process was also obtained when mycobacterium 301 was cultured on nitrogen-free medium without yeast autolyzate but with simultaneous inoculation of P. radiobacter and F. fulvum (4.22 mg of nitrogen per 1 g of alcohol consumed). These accompanying organisms apparently also supply this microorganism with some supplementary growth factors.

These experimental variants showed incontrovertibly that the presence of supplementary growth factors is indeed required for the successful growth of mycobacterium 301, but they did not reveal the differences between the effect of yeast autolyzate and of accompanying bacteria. This difference was determined in the next variant

TABLE 2

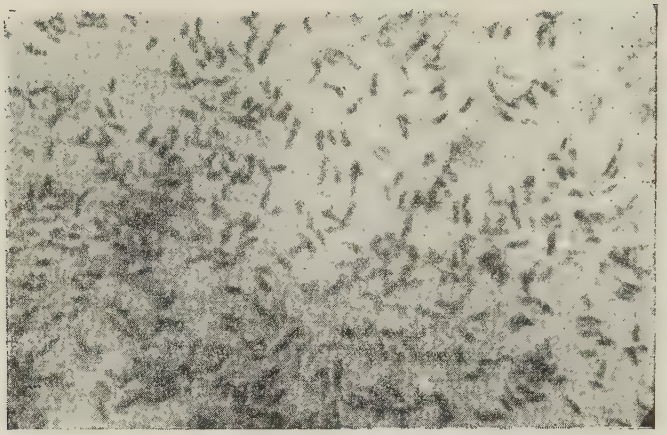
Nitrogen Fixation by Mycobacterium 301 on Medium with Ethanol and Yeast Autolyzate in Pure Culture and in Mixed Culture with Accompanying Bacteria

| Nutrient medium | Cultures | Nitrogen fixed in mg N per 30 ml of medium | Efficiency of nitrogen fixation in mg per g of ethanol consumed | Growth rate in units of optical density |
|---|---|---|---|---|
| Nitrogen-free, without carbon source | Mycobacterium 301 | 0.0 | – | 0.020 |
| | | 0.0 | – | 0.019 |
| | | 0.0 | – | 0.020 |
| Nitrogen-free + 0.225 g of ethanol | Mycobacterium 301 | 0.0 | – | 0.021 |
| | | 0.0 | – | 0.020 |
| The same | Mycobacterium 301 + | 1.0 | 4.44 | 0.420 |
| | +P. radiobacter + | 0.7 | 3.11 | 0.415 |
| | +F. fulvum | 0.0 | – | 0.050 |
| Medium with 0.8 mg of of yeast autolyzate nitrogen without ethanol | Mycobacterium 301 | 0.0 | – | 0.056 |
| Medium with 0.8 mg of yeast autolyzate nitrogen + 0.224 g of ethanol | Mycobacterium 301 | 1.23 | 5.42 | 0.264 |
| | | 1.30 | 5.78 | 0.285 |
| | | 0.66 | 2.93 | 0.164 |
| The same | Mycobact. 301 + P. radiobacter | 2.34 | 10.4 | 0.260 |
| | | 2.54 | 11.29 | 0.298 |
| The same | Mycobact. 301 + F. fulvum | 2.00 | 8.89 | 0.330 |
| | | 2.11 | 9.38 | 0.415 |
| The same | Mycobact. 301 + P. radiobacter + F. fulvum | 2.93 | 13.02 | 0.450 |
| | | 3.12 | 13.86 | 0.525 |

of the same experiment, in which mycobacterium 301 was cultured on nitrogen-free medium containing ethyl alcohol and yeast autolyzate, but into which the accompanying bacteria were also inoculated. With the additional inoculation of P. radiobacter, the efficiency of nitrogen fixation rose to 10.84 mg per 1 g of alcohol consumed, while with the additional inocuation of F. fulvum it rose to 9.13 mg. When both of these accompanying organisms were additionally inoculated, the nitrogen fixing efficiency on the medium with yeast autolyzate proved to be even higher, constituting 13.44 mg per 1 g of alcohol consumed, as is easily established from the data presented in Table 2.

The data presented show that, in the presence in the medium of yeast autolyzate and accompanying bacteria, mycobacterium 301 was better supplied with the supplementary growth factors which it requires. Some growth factors are evidently lacking both in yeast autolyzate and in the secretions of the accompanying bacteria. Only when both yeast autolyzate and the secretions of the accompanying bacteria are present in the medium is there a full complement of the vitamins required by the given microorganism and possibly of certain amino acids which may be present in small doses in yeast autolyzate. Since in this case the nitrogen fixing activity of the microorganism becomes very high, it would be of great interest to establish precisely which vitamins or amino acids are required for nitrogen fixation. This is the work in which we are presently engaged.

On the basis of the results of the experiments given, it must be regarded as established that the accompanying bacteria are fulfilling a double function in mixed cultures of mycobacterium 301 growing on glucose. They supply this bacterium both with supplementary growth factors and with carbon sources which are produced when they split glucose into simpler products of the type of ethyl alcohol and acetic or lactic acids. It is due to the utilization of these compounds that the bacterium grows and fixes atmospheric nitrogen. When splitting glucose, the accompanying bacteria utilize part of the glucose themselves. Therefore, the nitrogen fixing activity of mycobacterium 301, expressed in milligrams per 1 g of glucose consumed, is in actual fact considerably greater, since only part of the glucose utilized is relegated to this bacterium.



Mycobacterium 301 under the microscope. Two-day culture stained with methylene blue. Magnification 900 ×.

All of the above-stated shows that mycobacterium 301 is of great scientific interest as a very unique and very active fixer of molecular nitrogen. We therefore present a description of the morphological and cultural characteristics of this bacterium below. In its outward form, it is a straight or slightly bent nonmotile rod with nonhomogeneously staining protoplasm. The presence of metachromatic granules which stain violet-red with methylene blue is observed in young cells. The cells frequently have swollen ends, are of different lengths, and have clearly pronounced branching which is not always observed. This bacterium stains Gram-negative.

When growing on meat-peptone agar, a 24-hour culture has the appearance of bent rods of various dimensions which stain unevenly with methylene blue (usually, the poles of the cells stain well). The dimensions of the cells are 0.5-0.8× 1.5-3.5 μ. The dimensions of the cells of a three-day culture on the same medium are 0.3 - 0.4×1.5-2.7μ. These dimensions remain the same up to the age of two months. When growing on liquid nitrogen-free medium with ethyl alcohol, the cells shorten to the dimensions of 0.5-0.7×1.4-1.75μ. Along with these, coccoidal cells with a diameter of about 0.55μ are aslo encountered (figure).

On meat-peptone agar, it grows slowly and forms semi-slimy, round, smooth, shiny, yellow or yellow-brown colonies. It only alkalizes but does not liquefy gelatin, gives an orange-yellow film on potato slices and a yellowish sediment on the bottom of meat broth, and rapidly produces nitrites in nitrate broth, although it does not grow on synthetic medium with nitrate. According to Krasil'nikov's Determinative Key (1949), we related it to the genus Mycobacterium, leaving the species designation undefined for the present. According to a number of characteristics, it is closely related to the species Mycobacterium flavum, but is not identical with it. According to Bergey's Manual (1945), it is closely related to the species Corynebacterium equi. Its physiological properties enable one to assume that it has not yet been described in detail, let alone studied, although it is of great scientific interest.

Inasmuch as, while studying the species composition of other mixed nitrogen-fixing cultures, we succeeded in isolating several more bacterial forms belonging to the genus Mycobacterium which also actively fix atmospheric nitrogen, it must be assumed that the nitrogen fixing ability is not an exclusive characteristic of mycobacterium 301 alone. And since mycobacteria, in turn, are widely distributed in soil, it is quite possible that they also play a substantial role in the enrichment of soil with nitrogen due to the assimilation of atmospheric nitrogen. The clearly pronounced requirement for supplementary growth factors and considerable fastidiousness with respect to carbon sources enables them to grow and to fix atmospheric nitrogen only in combination with other bacteria which are encountered both in the soil and in the rhizospheres of agricultural plants. Therefore, the further detailed study of this group of nitrogen-fixing bacteria is of great scientific interest.

## SUMMARY

1. A new nitrogen-fixing species of a mycobacterium closely related to Mycobacterium flavum has been isolated from turf-podzol soils. This species, designated as mycobacterium 301, is very productive in fixing molecular nitrogen in association with bacteria such as Pseudomonas radiobacter and Flavobacterium fulvum, a pure culture of which cannot by themselves assimilate this form of nitrogen. It assimilates 10-13 mg molecular nitrogen per 1 g of glucose consumed when cultured together with the accompanying bacteria, while in a pure glucose culture it does not develop because it cannot assimilate glucose as a carbon source.

2. In glucose cultures the concomitant bacteria supply mycobacterium 301 with available carbon sources formed as a result of incomplete oxidation of glucose, as well as with additional growth factors.

3. In acetate and ethanol cultures they likewise supply the mycobacterium with additional growth factors and enhance its nitrogen fixing activity 2-3-fold versus the pure culture of this bacterium grown in a nitrogen-free medium with yeast autolyzate. The chemical nature and mechanism of the effect on nitrogen fixation exerted by the additional growth factors are under study.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**All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.**


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# STUDY OF THE PHOSPHORUS COMPOUNDS IN SOME SPECIES OF ACTINOMYCETES

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The data existing in the literature concerning the phosphorus metabolism of actinomycetes is far from complete [Demyanovskaya and Belozerskii (1954); Biffi, et al. (1954); Guberniev, et al. (1956a, b); Mikhailovskaya (1956); Belozerskii and Naumova (1957, 1958); Doskocil, et al. (1958); Zaitseva and Orlova (1959)]. In some representatives of this group of microorganisms, polyphosphates were found, the metabolism of which was often related to the presence of volutin granules [Ebel, et al. (1958); Guberniev, et al. (1959); Guberniev, et al. (1959); Zaitseva and Mikhailova (1959)].

In the present work, an attempt was made to answer the question: Are polyphosphates constant components of all species of actinomycetes and, in the formation of volutin granules, is their participation always necessary?

## MATERIALS AND METHODS

The following species of actinomycetes were used for the investigation: Actinomyces aureofaciens strains 11 and 112, Actinomyces streptomycini strains LS-1 and LS-3, Actinomyces globisporus var. roseus, Actinomyces rimosus, Actinomyces griseus No. 15, and Proactinomyces citreus.

The inocula of Actinomyces aureofaciens strain 11 and Actinomyces rimosus were grown on medium with corn extract (1%) and starch (2%), while A. aureofaciens strain 112 was grown on synthetic medium (Belozerskii and Naumova, 1958). The inoculum cultures of A. globisporus var. roseus, A. griseus No. 15 and P. citreus were grown on Czapek's solid medium, A. streptomycini strain LS-1 on soy bean medium with glucose, and strain LS-3 on pea agar.

The main mass of mycelium of all of the actinomycete species required for analysis was grown from inoculum obtained by the method described above, on shakers at 27-28 C. In this case, A. aureofaciens strains 11 and 112, A. griseus No. 15 and P. citreus were cultured on synthetic medium with ammonium citrate (0.24%), starch (2%) and $K_2HPO_4$ (0.25-0.5%). For A. streptomycini strain LS-3, the same medium was used, but twice as much ammonium citrate was added to it, the starch was replaced by 2% glucose, and the content of $K_2HPO_4$ was increased by 1%. In addition to this, cultures of A. griseus No. 15, P. citreus, and A. globisporus var. roseus were grown on a medium of the following composition (in %): corn extract—0.5; glucose—1; starch—1.5; $(NH_4)2SO_4$—0.35; NaCl—0.5; $CaCO_3$—0.5; pH of medium 6.8-6.9. An analysis of the phosphorus compounds of corn extract showed that 1 g contained 26.5 mg of total phosphorus, of which about 45-50% was orthophosphate, whereas the remaining phosphorus belonged to phytin. The mycelia of A. streptomycini strain LS-1 and A. rimosus were grown on the synthetic medium described by Severin and Gorskaya (Belozerskii and Naumova, 1957).

Thus, when growing different actinomycetes, the above-indicated media contained different amount of phosphate which, for some cultures, varied from 0.25 to 1%. The addition of such a large quantity of phosphate (1%) to the medium was necessary in order to be completely certain that, if the given organism did not contain polyphosphates, they were absent at high phosphorus contents of the medium as well. It is possible that in some cases

TABLE 1

Content of Various Forms of Phosphorus in μg per 1 g of Dry Weight in Actinomycetes Grown on Defined Medium

| Extracts | A. aureofaciens strain 11, 72-hr growth on medium with 0.25% $K_2HPO_4$ | | | | | | A. griseus No. 15, 24-hr growth on medium with 0.25% $K_2HPO_4$ | | | | | | 25-hr growth on medium with 0.5% $K_2HPO_4$ | | | | | | P. citreus, 25-hr growth on medium with 0.5% $K_2HPO_4$ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $P_{op}$ | $P_7$ | $P_{30}$ | $P_{pp}$ | $P_{na}$ | $P_{tot}$ | $P_{op}$ | $P_7$ | $P_{30}$ | $P_{pp}$ | $P_{na}$ | $P_{tot}$ | $P_{op}$ | $P_7$ | $P_{30}$ | $P_{pp}$ | $P_{na}$ | $P_{tot}$ | $P_{op}$ | $P_7$ | $P_{30}$ | $P_{pp}$ | $P_{na}$ | $P_{tot}$ |
| 1% trichloro-acetic acid at 0 C | 2456 | 2845 | 2862 | — | — | 3743 | 335 | 467 | 550 | 49 | — | 800 | 7200 | 7600 | 7750 | — | — | 8300 | 92 500 | 93 060 | 93 060 | — | — | 93 830 |
| $NaClO_4$, 0 C | 112 | 902 | 1056 | 636 | — | 1884 | следы | 31 | 60 | — | — | 200 | 530 | 525 | 525 | — | — | 700 | 1136 | 1352 | 1368 | 200 | — | 1402 |
| NaOH*, 0 C | — | 5591 | 7719 | 3463 | 5499 | 12 087 | — | 334 | 1589 | — | 2971 | 4050 | — | 360 | 1000 | — | 3526 | 3800 | — | 90 | 181 | — | traces | 254 |
| 5% $HClO_4$, 100 C, 30 min | — | — | — | — | — | — | 1938 | — | — | — | 5585 | 10 512 | 2750 | — | — | — | 3750 | 5500 | — | — | — | — | — | 268 |
| Original culture | — | — | — | — | — | — | | | | | | 16 200 | | | | | | 29250 | — | — | — | — | — | 94 500 |

Note: $P_{op}$) orthophosphate; $P_7$) phosphorus after 7-min hydrolysis with 1 N HCl in boiling water bath; $P_{30}$) after 30-min hydrolysis under the same conditions; $P_{pp}$) polyphosphate; $P_{na}$) nucleic acid phosphorus; $P_{tot}$) total phosphorus.

* Due to the identity of their composition, the analytical data of two alkaline extractions (pH 9-10 and pH 12) were combined into a single NaOH column.

TABLE 2

Content of Various Forms of Phosphorus in μg per 1 g of Dry Weight of Mycelium of Actinomycetes Grown in Medium Containing Corn Extract

| Extracts | A. griseus No. 15, 27-hr growth | | | | | A. globisporus var. roseus, 24-hr growth | | | | | P. citreus, 67-hr growth | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $P_{op}$ | $P_7$ | $P_{30}$ | $P_{na}$ | $P_{tot}$ | $P_{op}$ | $P_7$ | $P_{30}$ | $P_{na}$ | $P_{tot}$ | $P_{op}$ | $P_7$ | $P_{30}$ | $P_{na}$ | $P_{tot}$ |
| 10% trichloroacetic acid at 0 C | 26 | 75 | 149 | — | 2490 | 34 | 80 | 178 | — | 2868 | traces | traces | 69 | — | 1000 |
| $NaClO_4$ at 0 C | traces | 64 | 149 | — | 1074 | 0 | 60 | 170 | — | 1634 | traces | traces | 39 | — | 334 |
| NaOH at 0C | — | 1084 | 3898 | 7080 | 9859 | — | 316 | 2826 | 6331 | 9036 | — | 128 | 378 | 715 | 1150 |
| 5% $HClO_4$ at 100 C, 30 min | 1668 | — | — | 5368 | 12 800 | 1209 | — | — | 5100 | 10 750 | 1500 | — | — | 3945 | — |
| Original culture | — | — | — | — | 28 500 | — | — | — | — | 26 500 | — | — | — | — | 11 700 |

the absence of polyphosphates may be the result of a low phosphorus content of the medium and not of the fact that the given organism cannot synthesize this group of compounds at all.

The actinomycete mycelium was separated from the medium by centrifugation, thoroughly rinsed with water, and dried with alcohol-ether. In those cases where the culture fluid contained chalk, it was first dissolved with an equivalent amount of 0.1 N HCl solution. Fractionation of material obtained in this manner was carried out according to the method of Langen and Liss (Kulaev, et al., 1960). Phosphorus was determined by the method of Behrenblum and Haines as modified by Weil-Malherbe and Green (1951) in all cases.

## RESULTS AND DISCUSSION

As seen from the data in Table 1, when a number of actinomycetes were grown on synthetic medium, we detected polyphosphates only in A. aureofaciens (in the salt and alkaline fractions). Moreover, the presence of polyphosphates in this species of actinomycete was found in both of the strains which we investigated, and they were identified by means of precipitating their barium salts (Langen and Liss, 1958).

We did not find polyphosphates in the other species of actinomycetes investigated when grown on the same medium.

The data in Table 1 show that even when the phosphorus content of the medium was very great A. griseus No. 15 and P. citreus accumulated it in the mycelium in the form of orthophosphate. Aside from this, it is striking that a large amount of phosphorus hydrolyzable by 1 N acid in 30 min was present in the acid-insoluble fraction of A. griseus No. 15 (both in the alkaline and in the hot perchloric extracts). In order to determine the nature of this phosphorus and its possible relation to polyphosphates, we carried out a chromatographic study of the hydrolytic products of the phosphorus compounds in these fractions according to Thilo, et al. (1956) (pH 1.6, 1 hr at 70 C). Under these hydrolysis conditions, high polymer polyphosphates should have been hydrolyzed to ortho- and trimetaphosphate. However, an analysis of the chromatograms obtained (for conditions of chromatography, see Kulaev, et al., 1960) showed the complete absence of trimetaphosphate among the hydrolysis products of both the alkaline fraction and the hot perchloric extract. At the same time, an easily moving compound with an Rf close to that of orthophosphate was found among the hydrolysis products of both of the fractions tested. On the chromatogram, it moved in the ascending current of Ebel's acid solvent immediately behind orthophosphate, and when developed with Haines reagent and ammonia vapors, differed from it in color of the spot.

Thus, as shown by the chromatographic investigation, this compound is evidently a product of the incomplete hydrolysis of the relatively easily hydrolyzable compounds of the acid-insoluble fraction, but is not of a polyphosphate nature. Neither can the phosphorus compounds which we found in the given case be related to the phosphorylated polysaccharides (Belozerskii and Naumova, 1957), the phosphorus of which is very difficult to hydrolyze.

In analyzing the data which we obtained for A. streptomycini strain LS-1 and A. rimosus grown on synthetic medium of a different composition, it was found that the distribution of phosphorus among the fractions of these species of actinomycetes was analogous to that in A. griseus and P. citreus. No polyphosphates were found in this case as well.

After this, an attempt was made to detect polyphosphates in these same cultures grown on media containing corn extract. It is well known that the addition of the latter to the medium causes a considerable change in the phosphorus metabolism of a number of fungi [Guberniev and co-workers (1959a, b); Kulaev, et al. (1959)]. The results obtained in this case are presented in Table 2. When they are examined, the complete absence not only of polyphosphates, but also of any significant amounts of orthophosphate, in the acid-soluble fraction attracts one's attention first of all. At the same time, a very large amount of stable phosphorus, probably belonging to phytin, was found in both acid-soluble fractions (1% TCA and salt). A large amount of relatively easily hydrolyzable phosphorus compounds was also found in the acid-insoluble fraction of these organisms. The latter, as in the case of the A. griseus culture grown on synthetic medium, split off orthophosphate during a 30-min hydrolysis. In order to determine the nature of these compounds, the mycelium of A. griseus No. 15 grown on medium with corn extract was used. The barium salts of its alkaline fractions were precipitated (Langen and Liss,

1958), and their analysis is given in Table 3, along with that of the centrifugate obtained following their removal. As seen from Table 3, we were unable to separate nucleic acids from the easily hydrolyzable phosphorus compounds by means of barium precipitation. The easily hydrolyzable phosphorus compounds cannot be related to polyphosphates, since they are not completely precipitated by barium. Even in that part which did precipitate with barium, only a very negligible part of the phosphorus split off during a 7-min hydrolysis with 1 N HCl.

Thus, when a number of actinomycetes were grown on medium containing corn extract, we were also unable to detect polyphosphates.

It is known from data in the literature that volutin granules can be found in the mycelium of some actinomycetes at certain stages of development [Ebel, et al. (1958); Zaitseva and Mikhailova (1959); etc.]. At the same time, according to current concepts polyphosphates are necessary components of volutin granules [Wiame (1947); Ebel (1952); Belozerskii (1955)]. It was therefore of interest to determine the presence of polyphosphates in those actinomycetes in which they have not yet been found, but which were grown under conditions favoring the accumulation of a large number of volutin granules.

TABLE 3

Content of Various Forms of Phosphorus in μg in Barium Salt from Alkaline Fraction of A. griseus No. 15 (μg P per 1 g dry weight of mycelium)

| Preparation | $P_7$ | $P_{30}$ | $P_{na}$ | $P_{tot}$ |
|---|---|---|---|---|
| Barium salt | 136 | 373 | 728 | 1280 |
| Centrifugate | 488 | 2852 | 5950 | 8680 |

For this purpose, we used a culture of A. streptomycini strain LS-3 which was grown on the synthetic medium described above having a very high phosphorus content (1%). The presence of volutin granules was judged by staining of the culture preparations with an aqueous solution of methylene blue (1:1000) following fixation of the material in Carnoy's fluid. The microscopic control of the development of the mycelium showed that volutin granules appeared in large numbers by the 96th hour of growth of the culture. Therefore, the following were used for the analysis of phosphorus compounds: 1) young, 24-hr mycelium with marked basophilia and absence of volutin; and 2) old, 96-hr mycelium with a large amount of volutin granules. When the data presented in Table 4 are examined, it can be seen that polyphosphates were absent in mycelia of both ages.

TABLE 4

Content of Various Forms of Phosphorus in μg per 1 g of Dry Weight of Mycelium following 24- and 96- Hour Growth of a Culture of Actinomyces streptomycini Strain LS-3

| Extracts | 24 Hr | | | | | 96 Hr | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $P_{op}$ | $P_7$ | $P_{30}$ | $P_{na}$ | $P_{tot}$ | $P_{op}$ | $P_7$ | $P_{30}$ | $P_{na}$ | $P_{tot}$ |
| 1% TCA at 0 C | 1400 | 1400 | 1600 | — | 1865 | 517 | 550 | 550 | — | 634 |
| $NaClO_4$ at 0 C | 100 | 97 | 124 | — | 250 | 59 | 102 | 115 | — | 345 |
| NaOH, pH 8-9 at 0 C | — | 190 | 640 | 1920 | 2206 | — | 150 | 365 | 700 | 907 |
| NaOH, pH 12 at 0 C | — | 180 | 550 | 1548 | 1956 | — | 204 | 580 | 1237 | 1577 |
| 5% $HClO_4$ at 100 C, 30 min | — | — | 1544 | 4866 | 10 372 | — | — | 1325 | 5070 | 11 700 |
| Original culture | — | — | — | — | 19 240 | — | — | — | — | 23 500 |

In order to confirm this fact, a chromatographic study was carried out on the barium precipitates obtained from all of the extracts of the 96-hr culture which we analyzed. The conditions of chromatography were the same as in the case where the barium precipitates of the mycelial extracts of A. griseus No. 15 were studied. The barium was removed from these precipitates by means of KU-2 cation exchange resin in the Na form part of the solutions obtained in this manner were immediately spotted on the chromatogram following evaporation under a stream of cold air, while part—in the case of acid-insoluble fractions—was subjected to preliminary hydrolysis according to Thilo, et al. (1956). As a result of this, it was established that polyphosphates

were completely absent in all of the fractions investigated. Some other phosphorus compounds were found in the alkaline extract and in the residue remaining after the removal of acid-soluble and alkaline fractions. After hydrolysis of acid-insoluble fractions according to Thilo, aside from orthophosphate some phosphorus compound which moved with an Rf close to that of pyrophosphate in Ebel's acid solvent was also found among the products formed. This compound lagged behind pyrophosphate somewhat under these conditions of chromatography, and when developed with Haines reagent and ammonia vapors differed from polyphosphates in color of the spot (bluer).

Thus, the same as yet unidentified phosphorus compounds which we had found in A. griseus No. 15 were apparently present in the acid-insoluble fractions from A. streptomycini strain LS-3. However, in both cases, the hydrolytic products of these compounds differed in their mobility on chromatograms.

When the data in Table 4 are examined, it can be noted that in the young, 24-hr culture, in distinction from the old one, there was a greater amount of orthophosphate and nucleic acids. Apparently, the basophilia of young actinomycete mycelium was, as is the case with other organisms (Belozerskii, 1947), associated with the presence of free or at least a very labile bound form of RNA extractable by weak alkali (pH 8-9) under our conditions. In the old, 96-hr culture, where there was a relatively lower content of orthophosphate and nucleic acids, the amount of total phosphorus increased considerably. Moreover, the difference between the amount of phosphorus in the original culture and its total content in all of the fractions which we studied showed a particularly great increase. Considering this fact in the light of the presence of large quantities of volutin in the given culture, it can be assumed that its production in this case is connected not with polyphosphates, but rather with some other phosphorus compounds. These compounds may possibly be phosphorylated polysaccharides and phospholipids which we did not study directly, or some other as yet unknown phosphorus compounds. Thus, the second part of our work shows that the nature of volutin in various organisms can apparently differ somewhat and that it would hardly be correct to regard polyphosphates as absolutely necessary for the production of volutin. The data which we obtained are evidence of the fact that the question of the nature of volutin cannot be regarded as conclusively solved.

## SUMMARY

1. The phosphorus compounds of the following species of actinomycetes were investigated: Actinomyces aureofaciens strains 11 and 112, Actinomyces streptomycini strains LS-1 and LS-3, Actinomyces globisporus var. roseus, Actinomyces rimosus, Actinomyces griseus No. 15, and Proactinomyces citreus. They were grown on media with various phosphorus contents.

2. Of all of the species of actinomycetes enumerated above, polyphosphates were found only in A. aureofaciens.

3. In other species of actinomycetes (A. griseus No. 15 and A. streptomycini LS-3) some relatively labile phosphorus compounds were found which were not of a polyphosphate nature and which were hydrolyzable by 1 N HCl at 100 C in 30 min.

4. Apparently, polyphosphates are not necessary components of volutin granules in actinomycetes.

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# SIGNIFICANCE OF PROTEINS IN THE VITAL ACTIVITY OF SOME ANAEROBES

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When Kondrat'eva and Manoilov were investigating the culture fluid of the nonpathogenic anaerobic microbe Clostridium sporogenes, they observed its inhibitory effect on the growth of sarcoma M-1 in experiments on animals (Manoilov, 1960).

The study of the metabolic properties of anaerobes is of definite interest in connection with the possibility of using them as producers of anticarcinogenic substances. One of the methods of investigation in this direction is the search for growth conditions of the microbes on media containing a minimal amount of the substances required by the anaerobes for their vital activity and the study of the peculiarities of their protein metabolism at the same time. As is well known, Kitt-Tarocci medium, consisting of meat-peptone broth and small pieces of animal tissues, is the most widely used for growing anaerobes. There is nothing in the literature to indicate the significance of the various ingredients of this medium for the growth and development of anaerobes. We therefore carried out investigations to study the role of the individual components of the medium in the development of some anaerobes.

C. sporogenes, which is a representative of the putrefactive anaerobic microorganisms, wasused as the main material in the investigation.

The following experiments were set up in order to determine the significance of the broth in Kitt-Tarocci medium. Instead of 10 ml of broth, 10 ml of each of various solutions of mineral compounds in distilled water was added to 2 g of cooked meat.

As the result of numerous investigations of combinations of mineral salts, it was established that there was considerable growth of C. sporogenes when a meat-peptone broth was replaced by mineral medium of the following composition (in %): NaCl-0.3; $K_2HPO_4$-0.15; $KH_2PO_4$-0.03; $FeCl_3$-0.05; $NA_2SO_4$-0.03; $MgSO_4$-0.03; $CaCl_2$--0.01; $ZnSO_4$-traces (pH 7.4-7.6). However, growth was only one-fifth to one-fourth that on the usual Kitt-Tarocci medium. This mineral medium, which we conditionally designated as "mineral base," was used in subsequent experiments to select nitrogen and carbon sources which could replace meat protein for C. sporogenes and satisfy its requirements for essential elements of nutrition and energy. A great variety of combinations of the following ammonium salts were tested: sulfate, phosphate, formate, acetate, citrate—with such organic compounds as glucose, sucrose and sodium formate, acetate, and citrate. In addition to this, experiments were set up in which alanine, arginine, aspartic acid, valine, glycine, glumatic acid, histidine, leucine, lysine, norleucine, methionine, serine, tyrosine, tryptophane, cystine, cysteine, and phenylalanine were added both separately and in various combinations with each other to the combinations indicated above. Investigations were also carried out with enzymatic casein hydrolyzate.

It was found that only the combinations of sodium formate with ammonium sulfate improved the growth of C. sporogenes somewhat. In our experiments with amino acids, there was no growth of C. sporogenes in the ab-

sence of whole protein. The use of such substances as adenosine triphosphate, succinic and fumaric acids, the B vitamin complex, ascorbic acid, and vitamin A, which increase biological oxidation, did not give positive results.

In order to determine the importance of various proteins of vegetable and animal origin, we used meat, the cooked egg white from a chicken egg, potato, curds, as well as casein and gelatin. Two g of each of these substances, and casein and gelatin in amount to make 1%, were placed in 10 ml of mineral base as well as in meat-peptone broth. Considerable growth of C. sporogenes was observed on all media containing meat-peptone broth—approximately the same as on ordinary Kitt-Tarocci medium. In the medium with mineral base, growth occurred only in those cases where meat was present. Consequently, the presence of complex protein compounds is not sufficient for the growth of the anaerobe under investigation; it requires the presence of either meat broth or of substances extracted from fresh meat.

Growth of Clostridium sporogenes on Various Media with and without Meat Extract (2000 cells per 1ml were added during inoculation)

| № | Experimental variant | No. of cells in thousands/1 ml medium | Amine nitrogen (mg %) | | | Increment of amine nitrogen in % of initial |
|---|---|---|---|---|---|---|
| | | | initial | final | increment | |
| 1. | Kitt-Tarocci medium | 205 000 | 79.5 | 178.0 | 98.5 | 124.0 |
| 2. | Meat-peptone broth, meat extract | 188 000 | 86.0 | 97.0 | 11.0 | 12.8 |
| 3. | Broth from Kitt-Tarocci medium | 196 000 | 95.4 | 99.5 | 3.8 | 3.9 |
| 4. | Meat from Kitt-Tarocci medium, mineral base | 42 000 | 20.7 | 90.5 | 69,8 | 345.8 |
| 5. | Washed meat, mineral base, meat extract | 4 500 | 26.7 | 157.8 | 137.1 | 491.0 |
| 6. | Washed meat, mineral base | 2,0 | 17.8 | 18.7 | 0.9 | 5.0 |
| 7. | Mineral base, meat extract | 2,0 | 26.5 | 26.8 | 0.3 | 1.1 |
| 8. | Meat-peptone broth | 2,0 | 77.0 | 78.5 | 1.5 | 1.9 |

In order to determine the significance of these substances for the vital activity of C. sporogenes, a series of experiments was set up in which meat extract prepared from beef according to the method of Severin and Meshkova (1950) was used. After the meat was extracted, it was repeatedly washed with warm distilled water, squeezed through linen; this washed meat was then used as protein free of extractable substances.

The following were tested in the experiments: Kitt-Tarocci medium of the usual composition, media consisting of 2 g of small pieces of meat from Kitt-Tarocci medium, or of washed meat, in 10 ml of mineral base. Aside from these media, ordinary meat-peptone broth and the broth poured off from Kitt-Tarocci medium were used. Each of the media was placed in four test tubes, and 2% meat extract was added to two of them. After sterilization, a culture of C. sporogenes was inoculated into one tube without extract and one with extract. The sterile media, similarly to the experimental ones (inoculated), had vaseline oil poured into them and they were kept in an incubator for two days. After plating, the number of bacteria growing was counted, and all media were subjected to further investigation. The oil was removed from their surfaces, and then the proteins were precipitated with 20% trichloracetic acid and were centrifuged. Amine nitrogen was determined in the centrifugate (according to Tsuverkalov's method) and the amino acids were also fractionated by the ascending paper chromatography method (Zaitseva and Belozerskii, 1953). Repeated chromatography of the media (to two times) in a mixture of butanol—acid—water (40:10:50) was employed for the separation of amino acids.

The results of amine nitrogen determinations and quantitative counts of the growth of C. sporogenes in the media given above are presented in the table. From the data presented, it is seen that the extractable substances are decidedly of great significance. No growth was observed in the medium with washed meat and mineral base. When meat extract was added to this medium, the bacteria began to grow, although the multiplication rate was 40 times less than in Kitt-Tarocci medium. When, instead of washed meat, the meat from Kitt-Tarocci medium was used, the growth of C. sporogenes increased tenfold. At the same time, in the broth poured off from the Kitt-Tarocci medium, where the extractable substances of meat are apparently present, as well as in meat-peptone broth to which meat extract was added, the growth of the microbe was just as intensive as in the Kitt-Tarocci medium itself. The results of the determinations of amine nitrogen showed that, in media with whole protein (var-

iants 1, 4, 5), it became highly proteolyzed and the amount of amine nitrogen increased by 120-500% in the different variants. There was a definite relationship between the increment in amine nitrogen and the number of bacterial cells developing. In Kitt-Tarocci medium, the percent increment in amine nitrogen was four times less than in mineral medium with washed meat and meat extract, and 2.8 times less than in medium consisting of mineral base and meat from Kitt-Tarocci medium. At the same time, the growth of C. sporogenes in Kitt-Tarocci medium was 45 times better than in the first, and 2.5 times better than in the second. The increment in amine nitrogen in these media was inversely proportional to the growth of C. sporogenes in them. This is probably explained by the fact that, in the presence of mineral base instead of broth, the proteolytic enzymes of the microbe act primarily on whole proteins due to the absence of compounds required for the growth of the microbe in the liquid part of the medium. Therefore, the fewer such substances there are in the medium, the more drastic is the splitting to which complex proteins are subjected.

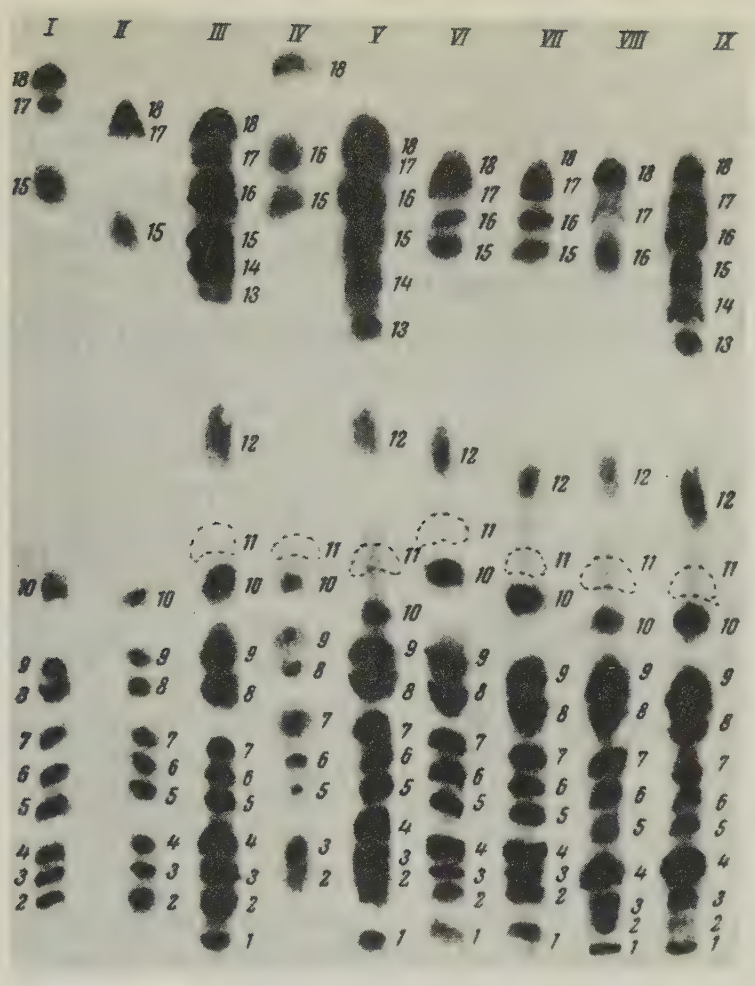
The results of the chromatographic fractionation of amino acids are presented in the figure. Chromatogram I characterizes the amino acid composition of meat extract, in which we were able to separate and identify 12 amino acids: lysine, histidine, arginine, aspartic acid, serine, glycine, glutamic acid, threonine, alanine, valine, leucine, norleucine. The amino acid composition of medium consisting of mineral base, washed meat, and meat extract (chromatogram II) was identical with that of meat extract. It was represented by the same 12 amino acids. The growth of the microbe in this medium (chromatogram III) was accompanied by profound proteolysis of protein. The additonal appearance of six more amino acids—cystine, proline, tyrosine, tryptophane, methionine, and phenylalanine—was evidence of this; in addition to this, more intense color of the spots of those amino acids which were already present in sterile medium was noted. This indicates that they increased in quantity. Following the growth of C. sporogenes in medium with mineral base and meat from Kitt-Tarocci medium (chromatogram V), an increase in the color intensity of the spots of amino acids present in the initial medium was also observed (chromatogram IV), as well as the appearance of six new ones: cystine, arginine, tyrosine, tryptophane, methionine, and leucine. A total of 18 amino acids was isolated and identified in it. Fifteen amino acids (chromatogram VIII) were detected in sterile Kitt-Tarocci medium. Following the growth of C. sporogenes in it, there was some increase in the amino acids present in the medium prior to inoculation and the appearance of three new amino acids corresponding to tryptophane, methionine, and valine (chromatogram IX). On chromatogram VI, the results of an analysis of the amino acids in sterile meat-peptone broth with meat extract are presented, while on chromatogram VII—an analysis of the same kind of broth following the growth of C. sporogenes in it. A comparison of these chromatograms shows that the amino acid composition of the medium did not change qualitatively during the course of growth of C. sporogenes and was represented by 16 amino acids.

Thus, the results obatined from the chromatographic analysis of the media show that amino acids not only do not disappear following the growth of C. sporogenes in them, but that they appear in large quantities. This is particularly true of media containing meat, where intensive hydrolysis of whole protein was observed, and where we demonstrated a nearly full complement of amino acids.

The change in amine nitrogen during the course of growth of C. sporogenes on Kitt-Tarocci medium was also studied after each 24-hr period for 20 days. An increment in amine nitrogen was observed during the first 120 hr; however, its intensity differed during this period: after 24 hr it constituted one-half of the maximal increment, while after 48 hr it was three-fourths of the maximal increment. Platings made to count the number of bacteria showed that the rise in amine nitrogen was connected with the most rapid rate of reproduction.

Aside from determinations of amine nitrogen in Kitt-Tarocci medium, quantitative tyrosine determinations were done daily as an indicator of the completeness of hydrolysis. Changes in amount of tyrosine are subject to the same regularities as changes in amine nitrogen.

In order to make a comparative evaluation of the results obtained, some of the experiments presented were set up with other species of anaerobes—with Clostridium histolyticus and Clostridium perfringens. As is well known, C. histolyticus has considerable proteolytic activity, while in C. perfringens it is less pronounced. Tests were conducted with both species of bacteria to determine the importance of meat extract for their growth. Changes in amine nitrogen and the amino acid composition of the media during growth of the given bacteria were also studied. They agreed to a considerable extent with the results obtained when C. sporogenes was studied. The growth of these anaerobes was also in direct relationship to the presence of meat extractables in the medium. The determination of amine nitrogen during the course of growth of C. histolyticus showed that its increase was approximately the same as in similar media during the growth of C. sporogenes, which indicates lines of similarity in




Chromatographic analysis of amino acids in sterile media and following the growth of Clostridium sporogenes. 1) Cystine; 2) lysine; 3) histidine; 4) arginine; 5) aspartic acid; 6) serine; 7) glycine; 8) glutamic acid; 9) threonine; 10) alanine; 11) proline; 12) tyrosine; 13) tryptophane; 14) methionine; 15) valine; 16) phenylalanine; 17) leucine; 18) norleucine. I) Meat extract, mineral base; II, III) washed meat, meat extract, mineral base (II-sterile, III-following growth); IV, V) meat from Kitt-Tarocci medium, mineral base (IV-sterile, V-following growth); VI, VII) meat-peptone broth, meat extract (VI-sterile, VII-following growth); VIII, IX) Kitt-Tarocci medium (VIII-sterile, IX-following growth).

their metabolism. In this case, the inversely proportional relationship between the amount of bacterial cells and the increment of amine nitrogen was also observed. The similarity in the metabolism of these anaerobes was confirmed by the data of the chromatographic analysis.

In experiments with C. perfringens, the regularities pertaining to growth rate were the same as for the other two microbes studied. However, the increase in amine nitrogen during the course of its growth was relatively small; it was approximately 2.5-3 times less than in the corresponding media following the growth of the other two anaerobes. Chromatographic analysis of the culture fluid showed that only two amino acids appeared in the medium consisting of mineral base and washed meat and in Kitt-Tarocci medium. The results obtained are evidence of the weaker proteolytic properties of C. perfringens as compared with the other two anaerobes.

## SUMMARY

1. Clostridium sporogenes does not grow in defined media containing various amino acids, carbohydrates, mineral salts, vitamin complexes, and activators of microbial growth and tissue respiration.

2. C. sporogenes can grow only in media containing proteins or the products of their partial hydrolysis.

3. The extractable substances of meat are very important for the growth of such anaerobes as C. sporogenes, Clostridium histolyticus, and Clostridium perfringens.

4. C. sporogenes and C. histolyticus possess high proteolytic activity and cause the breakdown of protein to free amino acids with a two- to sixfold increase in amine nitrogen, depending on the composition of the medium.

5. Proteolytic properties are considerably less pronounced in C. perfringens, and the increase in amine nitrogen is 2.5-fold at best.

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# PRODUCTION OF VITAMIN $B_{12}$ BY *Actinomyces olivaceus* ON DEFINED MEDIUM

I. V. Konova and A. I. Borisova

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January-February, 1961

Many actinomycetes produce vitamin $B_{12}$ in addition to antibiotics. Actinomyces olivaceus, which produces hardly any antibiotic, is especially useful as a producer of vitamin $B_{12}$.

The data in the literature concerning the biosynthesis of $B_{12}$ by A. olivaceus in connection with the process of growth of the producer and the conditions for its cultivation are not very numerous, and in the majority of cases were obtained during the cultivation of the actinomycete on media containing an undefined composition of components of the type of corn extract, etc. [Hall and co-workers (1953); Pfeifer and co-workers (1954); Makarevich and co-workers (1958) et al.].

Synthetic media were employed in the work of Gangully and Roy (1953); in this case, the inoculum used was grown in the presence of casein hydrolyzate and meat extract; the maximal vitamin yield was 0.72 μg/ml.

In the present work, the production of vitamin $B_{12}$ by a culture of A. olivaceus was studied during growth on synthetic medium.*

## METHODS

The A. olivaceus culture was maintained on pea agar. The experiments were carried out at 26-28 C on liquid media, the composition of which is given below. Cultivation was on shakers rotating at an average of 120-160 rpm, in 250 ml Erlenmeyer flasks containing 40 ml volumes of medium. In some cases, the experiments were set up under stationary conditions. The amount of vitamin was determined by the microbiological and bioautographic methods with a mutant of E. coli strain 113-3 sensitive to methionine [Chaikovskaya and Druzhinina. (1957); Ierusalimskii and co-workers (1959)].

The test sample (mycelium, centrifugate of culture fluid or whole culture fluid) having a pH of 4.5-5, in the presence of $NaNO_2$ (0.75 ml of a 5% solution per 15 ml of test liquid), was autoclaved at 0.5 atm for 15 min.

Chromatographic fractionation was carried out in secondary butanol saturated with water, at alkaline ($NH_4OH$) and acid ($CH_3COOH$) pH in an atmosphere of NaCN (Verkhovtseva et al., 1956). The absolute dry weight of the mycelium was determined following centrifugation and washing of the sediment with 5% $CH_3COOH$ and water; the glucose content of the medium was determined according to Bertrand, and ammonium nitrogen was determined by the diffusion method; the pH of the medium was determined potentiometrically. Vegetative mycelium served as the inoculum; it was used in the amount of 2.5 % by volume, usually after 48 hr of growth in medium of the same composition as the experimental medium. In order to obtain inoculum, spores of the actinomycete from pea agar were used.

## EXPERIMENTAL

1. Selection of medium. After testing several synthetic media, Dulaney's medium (1953) was used as the

* The work was carried out under the direction of Prof. V. N. Shaposhnikov and Prof. N. D. Ierusalimskii.

TABLE 1

Growth and $B_{12}$ Production of A. olivaceus on Defined Medium with Various Nitrogen Sources

| Nitrogen source | Expt. No. 1 (fourth day) | | | Expt. No. 2* (fourth day) | | |
|---|---|---|---|---|---|---|
| | pH | mycelium weight, g/100 ml | $B_{12}$ μg/100 ml | pH | mycelium weight, g/100 ml | $B_{12}$ μg/100 ml |
| $(NH_4)_2SO_4$ | 5.4 | 0.11 | 7.0 | 5.04 | 0.35 | 11 |
| $(NH_4)NO_3$ | 6.3 | 0.10 | 8.0 | — | — | — |
| $NaNO_3$ | 7.3 | 0.08 | 7.0 | 7.68 | 0.50 | 19 |
| $NH_4$ succinate | — | — | — | 7.32 | 0.59 | 48 |

* In experiment No. 2, the inoculum was prepared on medium containing 2.5% rye spent liquor and was added in the amount of 5%. The experimental medium contained 0.25% $CaCO_3$.

TABLE 2

Effect of the Culture Conditions of the Inoculum on the Growth and $B_{12}$ Production of A. olivaceus

| Expt. No. | Inoculum obtained from medium | Growing time, hours | Maximal amount | |
|---|---|---|---|---|
| | | | mycelium, g/100 ml | $B_{12}$, μg/100 ml |
| 1 | With spent liquor | 48 | 0.48 | 81 |
| | Defined * | 96 | 0.52 | 58 |
| 2 | With spent liquor | 48 | 0.40 | 100 |
| | Defined | 96 | 0.40 | 97 |

* 10 mg/liter of $MnSO_4$ was added to the medium without spent liquor.

base; at first, it was used with hardly any changes (0.6 mg % $CoCl_2$ was used and $CaCl_2$ was omitted). Changes were introduced during the course of the work which are noted in the presentation of the results. When several nitrogen sources were tested in this medium (taken in amounts equivalent to 100 mg N/100 ml of medium), the best results were obtained with ammonium succinate. Mineral sources of nitrogen gave equally low amounts of mycelium and vitamin* (Table 1). When $(NH_4)_2SO_4$ was used, the physiological acidity of the salt had an unfavorable effect. When the medium was neutralized, the growth of the actinomycete improved noticeably. When $NaNO_3$ was added, an increase in the mycelial mass and amount of vitamin was observed when the inoculum was prepared in medium containing 0.5-2.5% rye spent liquor.

Ammonium succinate, which gave satisfactory yields of mycelial mass and vitamin, was subsequently used as the nitrogen source. Nearly equal maximal amounts of mycelial mass and vitamin $B_{12}$ were obtained in experiments where the inoculum used was grown on medium containing rye spent liquor (2.5% by volume) or without it (Table 2).

The actinomycete utilized both the nitrogen and the carbon chain of ammonium succinate, which was confirmed by the results of experiments where Na succinate was added to medium containing $(NH_4)_2SO_4$ (Fig. 1).

As seen from Fig. 1, in the case where sodium succinate was present in the medium, a sharp rise in pH occurred as compared with the control.

Alkalization took place due to the sodium ions released as the result of the consumption of the carbon portion of the salt.

* In the given case and in the following presentation, vitamin $B_{12}$ is understood to designate the total amount of vitamin detectable with E. coli by the plate method.

TABLE 3

Growth and $B_{12}$ Production of A. olivaceus on Defined Medium * Containing Various Amounts of Glucose

| Amount of glucose in medium, % | 48 Hours | | | 96 Hours | | | Sugar in medium, % |
|---|---|---|---|---|---|---|---|
| | pH | mycelium weight, g/100 ml | $B_{12}$, μg/100 ml | pH | mycelium weight, g/100 ml | $B_{12}$, μg/100 ml | |
| 0 | 7.40 | 0.057 | 3.9 | 8.15 | 0.042 | 15 | — |
| 1 | 6.20 | 0.277 | 12.5 | 8.65 | 0.263 | 122 | 0 |
| 2 | 6.11 | 0.478 | 12.3 | 7.66 | 0.390 | 106 | 0.12 |
| 3 | 6.11 | 0.553 | 18.0 | — | 0.402 | 102 | 0.67 |
| 4 | 6.11 | 0.773 | 10.0 | 5.88 | 0.433 | 107 | 2.13 |

*The medium contained $K_2HPO_4$ – 1.2 g; $KH_2PO_4$ – 0.63 g; $CoCl_2 \cdot 6H_2O$ – 6 mg; $CaCO_3$ – 5 g; and $MnSO_4$ – 10 mg per liter.

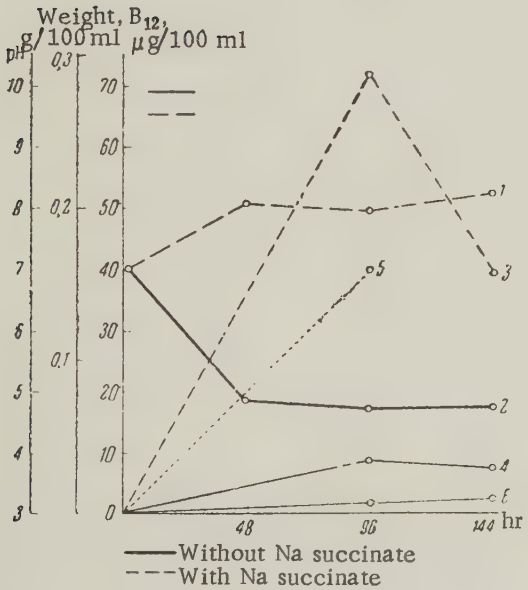



Fig. 1. pH of the medium, weight of mycelium, and $B_{12}$ production on media with and without Na succinate. 1, 2) pH of medium; 3, 4) weight of mycelium; 5, 6) $B_{12}$.

The consumption of succinic acid had a favorable effect on the growth of the producer and vitamin biosynthesis.

It can be assumed that the positive effect of ammonium succinate is explained not only by the buffering action of this salt, but also by the positive effect of succinic acid on the growth of the organism, as well as by its specific effect on the biosynthesis of $B_{12}$.

However, when ammonium succinate was used as the sole source of nitrogen and carbon, poor growth of the actinomycete was observed. Considerably larger amounts of biomass of the producer and of the vitamin accumulated in the presence of glucose (1-4%) (Table 3). As can be seen, 1% proved to be sufficient. We later used 2% glucose in order to avoid possible sugar deficiency.

In the later experiments, we used a medium of the following composition: 1 liter of distilled water containing 20 g of glucose, 5 g of NaCl, 1.2 g of $K_2HPO_4$, 0.63 g of $KH_2PO_4$, 1 g of $MgSO_4 \cdot 7\ H_2O$, 10 mg of $FeSO_4 \cdot 7\ H_2O$, 10 mg of $ZnSO_4 \cdot 7\ H_2O$, 10 mg of $MnSO_4$, 15 mg of $CoCl_2 \cdot 6\ H_2O$, 5.4 g of $NH_4$ succinate, 5 g of $CaCO_3$. The chalk and phosphate and glucose solutions were sterilized separately. The initial pH of the medium was 6.8-7.0.

2. The biosynthesis of vitamin $B_{12}$ during the course of growth on synthetic medium. When A. olivaceus was grown on synthetic medium in a stationary culture, using 250 ml Erlemeyer flasks with 80 ml of medium, it produced a fairly thick mycelial pellicle and separate floc-like submerged colonies.

The actinomycete synthesized the vitamin actively in stationary culture, producing approximately 0.08 mg per 100 ml of culture by the ninth day.

The mycelium, the weight of which averaged about 0.4 g per 100 ml of medium, contained approximately 250 μg of vitamin $B_{12}$ per 1 g.

In relatively anaerobic conditions (during growth in tall test tubes with a diameter of 20 mm and a liquid layer of 27 cm in height) which are unfavorable for the growth of the actinomycete, 0.017-0.019 g of mycelium accumulated per 100 ml of medium, and the culture contained 0.66-0.75 μg per 100 ml of the vitamin; the mycelium contained about 39 μg per 1 g.

TABLE 4

Vitamin $B_{12}$ Content of an A. olivaceus Culture

| Time, hr | Flask No. | pH of medium | mycelium weight, g/100 ml | $B_{12}$ total amount in culture, µg/100 ml | $B_{12}$ in mycelium from 100 ml, µg | $B_{12}$ in mycelium % | $B_{12}$ in culture fluid µg/100 ml | $B_{12}$ in culture fluid % |
|---|---|---|---|---|---|---|---|---|
| | | Experiment No. 2 | | | | | | |
| | 1 | 6.4 | 0.001 | 0.19 | 0.19 | 100 | traces | — |
| 24 | 2 | 6.6 | 0.004 | 0.15 | 0.15 | 100 | „ | — |
| | Av. | 6.5 | 0.0025 | 0.17 | 0.17 | 100 | „ | — |
| | 1 | 6.1 | 0.067 | 4.04 | 4.04 | 100 | „ | — |
| 48 | 2 | 6.1 | 0.112 | 3.48 | 3.48 | 100 | „ | — |
| | Av. | 6.1 | 0.090 | 3.75 | 3.50 | 100 | „ | — |
| | 1 | 5.7 | 0.342 | 22.88 | 21.08 | 92.1 | 1.8 | 7.9 |
| 72 | 2 | 6.1 | 0.133 | — | — | — | 2.1 | — |
| | Av. | 5.9 | 0.233 | | | | | |
| | 1 | 7.4 | 0.721 | 68.5 | 66.8 | 97.5 | 1.7 | 2.5 |
| 96 | 2 | — | 0.563 | 9.94 | 9.54 | 96.0 | 0.4 | 4.0 |
| | Av. | | 0.642 | | | | | |
| | 1 | 8.73 | 0.658 | — | — | — | 12.2 | |
| 120 | 2 | 7.0 | 0.514 | — | — | — | 5.5 | |
| | Av. | 8.86 | 0.586 | | | | | |
| | 1 | 9 | 1.0 | 123 | 27.6 | 22.4 | 95.4 | 77.6 |
| 168 | 2 | 9 | 0.326 | — | — | — | 50.7 | |
| | Av. | 9 | 0.663 | | — | — | | |
| | | Experiment No. 3 | | | | | | |
| 48 | 1 | 6.5 | 0.060 | 4.1 | 4.1 | 100 | traces | — |
| 72 | 1 | 6.2 | 0.37 | 25.7 | 25.7 | 100 | „ | — |
| 96 | 1 | 8.5 | 0.432 | 286.3 | 149.5 | 52.2 | 48.3 | 47.8 |
| 120 | 1 | 9.0 | 0.360 | 242.4 | 102 | 42.1 | 80.4 | 57.9 |
| 168 | 1 | 9 | 0.229 | 237 | 25.2 | 24.5 | 179 | 75.5 |
| | | Experiment No. 4 | | | | | | |
| | 1 | 6.6 | 0.092 | 7.83 | 7.78 | 99.3 | 0.05 | 0.7 |
| 48 | 2 | 6.6 | 0.135 | 13.42 | 13.39 | 99.8 | 0.023 | 0.2 |
| | Av. | 6.6 | 0.114 | 10.62 | 10.59 | 99.6 | 0.038 | 0.4 |
| | 1 | 8.4 | 0.556 | 74.5 | 54.4 | 73.0 | 20.1 | 27.0 |
| 96 | 2 | 8.4 | 0.562 | 59.1 | 58.5 | 99.0 | 0.3 | 1.0 |
| | Av. | 8.4 | 0.559 | 66.6 | 56.5 | 86.0 | 10.2 | 14.0 |
| | 1 | 9 | 0.321 | 158 | 9 | 5.7 | 149 | 94.3 |
| 168 | 2 | 9 | 0.302 | 168 | 9 | 5.4 | 159 | 94.6 |
| | Av. | 9 | 0.311 | 163 | 9 | 5.5 | 154 | 94.5 |

The production of vitamin $B_{12}$ on synthetic medium during the growth of A. olivaceus in submerged culture is shown in Tables 4 and 5 and in Fig. 2, where changes in mycelial weight and amount of vitamin $B_{12}$ in the whole culture (mycelium + culture fluid) and individually in the mycelium and culture fluid are represented for consecutive intervals of time during the course of growth of A. olivaceus.*

* In Table 5, where calculations are made for two experiments, changes in weight and vitamin in the culture in each experiment are expressed in percent with respect to the maximum amount of mycelium and vitamin noted in the given experiment.

TABLE 5

Changes in Weight of Mycelium and Amount of Vitamin $B_{12}$ in Consecutive Time Intervals during the Course of Growth of A. olivaceus

| Time, hr | Flask No. | Experiment No. 1 | | | | Experiment No. 2 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | weight | | $B_{12}$ | | weight | | $B_{12}$ | |
| | | g/100 ml | % of maximum amount | g/100 ml | % of maximum amount | g/100 ml | % of maximum amount | g/100 ml | % of maximum amount |
| 0—24 | 1 | +0.06 | 9 | +0.5 | 0.3 | +0.001 | 0.14 | + 0.19 | 0.14 |
| | 2 | +0.09 | 15.1 | +2.8 | 1.0 | +0.004 | 0.71 | — | — |
| 24—48 | 1 | +0.149 | 22.9 | + 0.6 | 0.3 | +0.066 | 9.13 | + 5.34 | 4.1 |
| | 2 | +0.102 | 17.1 | + 17.1 | 6.3 | +0.108 | 19.2 | + 4.39 | 2.6 |
| 48—72 | 1 | +0.201 | 30.9 | + 29.8 | 15.7 | +0.275 | 38.2 | +14.5 | 11.5 |
| | 2 | +0.461 | 77.3 | +116.1 | 41.0 | +0.021 | 3.7 | + 9.1 | 5.4 |
| 72—96 | 1 | +0.293 | 45.1 | + 32.1 | 16.9 | +0.379 | 52.2 | +52.5 | 4.0 |
| | 2 | +0.007 | 1.2 | + 11.0 | 4.0 | +0.430 | 76.2 | — 2.4 | 1.4 |
| 96—120 | 1 | —0.07 | 10.8 | +109 | 57.4 | —0.063 | 8.8 | +57.5 | 43.8 |
| | 2 | +0.17 | 28.5 | +125 | 45.9 | —0.049 | 8.7 | +26.4 | 15.3 |
| 120—144 | 1 | —0.266 | 41 | — 8 | 4.2 | — | — | — | — |
| | 2 | —0.248 | 41.6 | — 16 | 5.9 | — | — | — | — |
| 120—192 | 1 | — | — | — | — | +0.342 | 33.4 | 0 | 0 |
| | 2 | — | — | — | — | —0.188 | 47.2 | +131.5 | 77.5 |
| 144—168 | 1 | —0.008 | 1.2 | + 26 | 13.7 | — | — | — | — |
| | 2 | —0.058 | 9.7 | — 69 | 25.4 | — | — | — | — |
| 168—192 | 1 | —0.183 | 28.2 | — 18 | 9.5 | — | — | — | — |
| | 2 | —0.155 | 26.0 | + 25 | 9.1 | — | — | — | — |

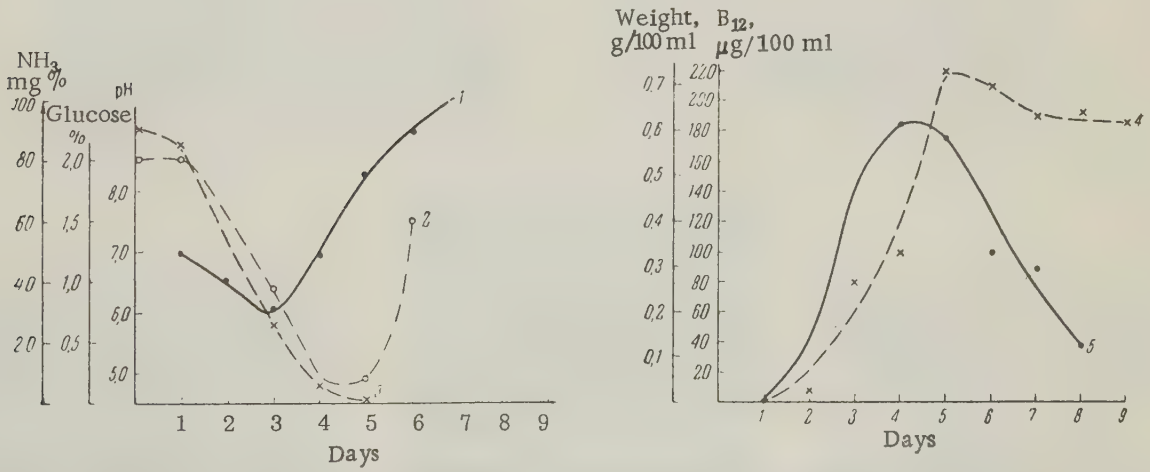


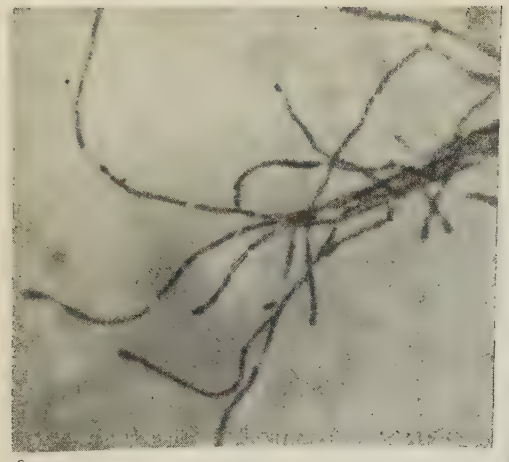

Fig. 2. Changes occurring in an A. olivaceus culture in the dynamics of growth of the organism. 1) pH; 2) $NH_3$ nitrogen; 3) glucose; 4) $B_{12}$; 5) weight of mycelium.

Growth was very poor during the first 24 hours, an average of about 4% of the maximal mycelial mass accumulating in this time; this figure fluctuated from 0.14 to 15% in individual experiments. In 48 hr, from 9 to 24%, or an average of 18%, of maximal weight of mycelium was observed.

The most vigorous growth occurred during the 2-4-day interval, the greatest increment being observed either on the 2-3rd day or on the 3-4th day, and sometimes the growth rate was nearly identical during these time intervals. The main mass of mycelium accumulated during this time.

The process of growth was accompanied by a high rate of utilization of the nutrient components of the medium. Thus, nitrogen which was added to the medium in the amount of 100 mg/100 ml and glucose (2%) were completely consumed by the fourth day. According to the data from qualitative chromatographic analysis (experiment No. 4), succinic acid was also completely consumed by the third day.

As a result of the vital activity of the actinomycete, the pH of the nutrient medium dropped to 6-5.5 or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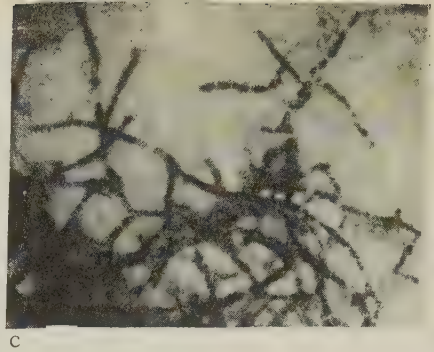
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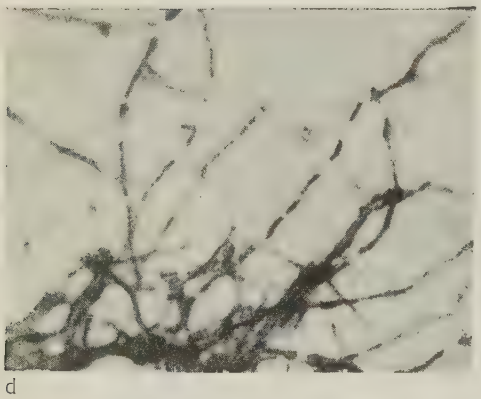
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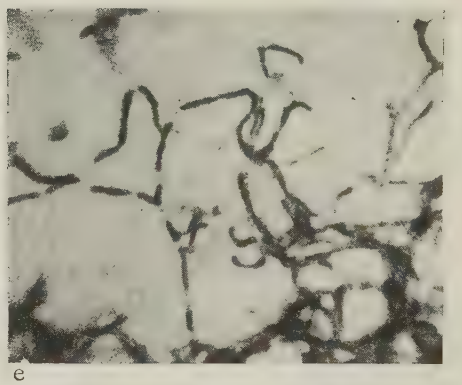
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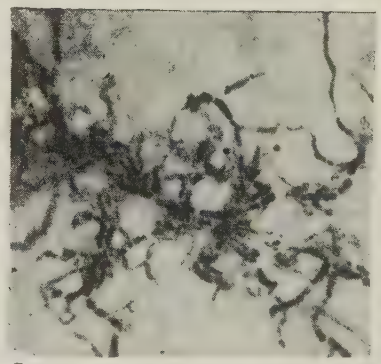
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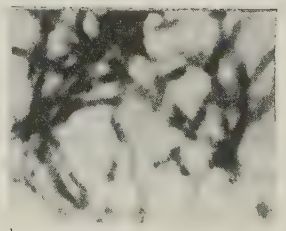
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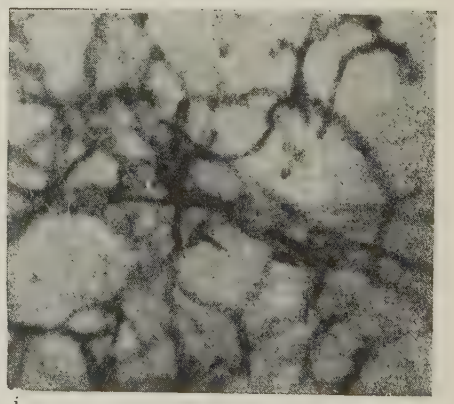
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Fig. 3. Morphology of A. olivaceus in the dynamics of growth (preparation stained with methylene blue). Magnification 2.5 thousand times. a, b) Second day; c, d) third day; e, f) fourth day; g, h) fifth day; i) seventh day.

somewhat lower by 72 hr. Subsequently, the pH rose to 9 or higher after 5 days during the period of mass autolysis.

When the culture was examined microscopically during its course of development (Fig. 3), microcolonies consisting of long intertwining hyphae with lateral branches could be observed as early as 24 hr. The protoplasm was homogeneous and basophilic; in some hyphae, differentiation of the basophilic protoplasm was observed. During the 48-72 hr period, the hyphae were thicker, and some had club-shaped swellings on the ends. Segmentation of the protoplasm, the basophilia of which decreased in some hyphae by the 72nd hour, was very pronounced.

The basophilia of the protoplasm decreased by the 96-120th hr, and sometimes even by the 72nd hr, and basophilic nuclear fragments became differentiated in it.

After 4-5 days, along with the finer, weakly basophilic hyphae there were also thick ones with branching filaments and signs of differentiation of the protoplasm. On the seventh, or sometimes on the 9-11th day, noticeable secondary growth was observed.

The production of vitamin $B_{12}$ by the A. olivaceus culture started during the first day of growth; after 24 hr, vitamin $B_{12}$ could be detected in amounts of from 0.14 μg to 2.8 μg per 100 ml, which constituted 0.1-1% of the maximal amount in the culture (or somewhat higher).

The amount of $B_{12}$ increased in each subsequent 24-hr period of growth of the culture (to 72-96 hr), and reached about 40% of maximum at the peak of the growth curve. As a rule, the greatest increase in the amount of vitamin in the culture occurred after the break in the growth curve, i.e., in the 96-120 hr interval. The amount of vitamin produced during this period of time averaged over 50%, and sometimes 70-90% of maximum. Thus, the greatest amount of vitamin was synthesized by the mycelium during the time when the growth decreased and autolytic processes began to prevail.

In some of our experiments lasting for 11-14 days, a further increase in the amount of $B_{12}$ in the culture was observed by this time; this probably took place due to secondary growth of the mycelium.

During the course of growth of A. olivaceus, vitamin $B_{12}$ was distributed between mycelium and the medium in the following manner: during the first phase (growth phase), nearly all of the vitamin was in the mycelium, and only 3-9%, or sometimes as much as 17%, was released into the culture fluid. After the fourth day, when mass autolysis began, the vitamin was rapidly released into the medium. At this time, the culture fluid contained 70-90% of the total amount of vitamin (Fig. 4).

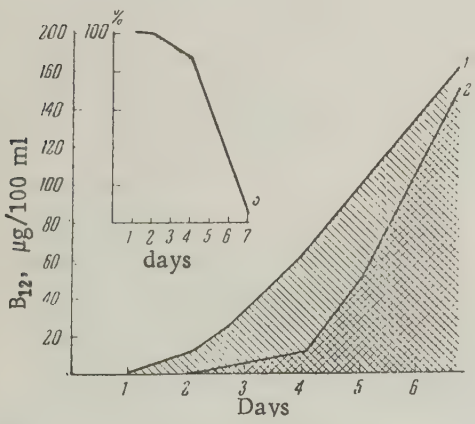


Fig. 4. Distribution of $B_{12}$ in an A. olivaceus culture. 1) $B_{12}$ in mycelium; 2) $B_{12}$ in culture fluid; 3) percent of $B_{12}$ in mycelium.

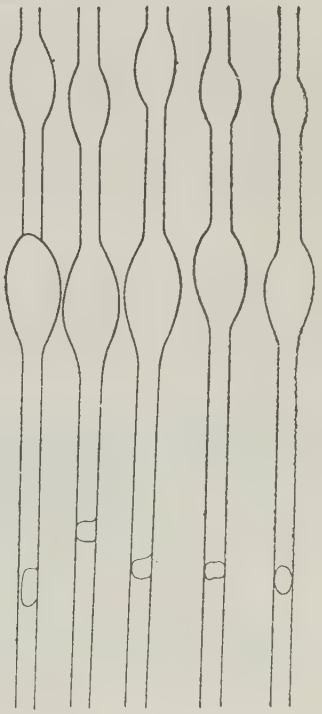

Fig. 5. Bioautogram of E. coli following chromatographic fractionation ($NH_4OH$) of samples from an A. olivaceus culture on defined medium. 1) Ascending chromatogram; 2) true $B_{12}$ spot. [Key nos. 1 and 2 omitted in Russian original—Publisher.]

When the mycelium was washed with 5% $CH_3COOH$ and water, the vitamin was washed out of the mycelium and was present in considerable quantities in both the wash liquids.

When the vitamin produced by A. olivaceus on our medium was fractionated chromatographically, aside from the spot of true $B_{12}$, two or three other forms of the vitamin were also detected on the bioautogram (Fig. 5). The $R_f$ of one of these forms was higher than that of true $B_{12}$, while the other two forms which were produced in minute quantities (one of which did not always develop on the bioautograpm) had a considerably lower $R_f$ than true $B_{12}$. True $B_{12}$ was predominantly produced (about 70%) in the A. olivaceus culture.

In conclusion, we should like to express our deep appreciation to Prof. V. N. Shaposhnikov and Prof. N. D. Ierusalimskii for directing the work.

## SUMMARY

1. On synthetic medium containing, in addition to mineral salts, 2% glucose and 0.54% ammonium succinate, a culture of Actinomyces olivaceus growing in submerged culture accumulates a maximum of 0.4-0.7 g of absolutely dry mycelium and 131-332 μg of vitamin $B_{12}$ per 100 ml of culture. The maximal vitamin content of the mycelium is 182-663 μg/g of mycelium.

2. Aside from true $B_{12}$, which comprises the greatest part (about 70%), A. olivaceus growing on synthetic medium produces other, as yet unidentified, forms of the vitamin as well.

3. Two phases can be distinguished during the growth of A. olivaceus on synthetic medium. The main mass of mycelium accumulates during the first phase, which lasts for 3-4 days. During this period, about 40% of the maximum amount of vitamin is synthesized in the culture. Nearly all of the vitamin is found in the mycelium. During the second growth phase of the culture, when there is practically no growth of the mycelium, more than half of the maximal amount of viatmin is snythesized. As a rule, this increase occurs at 24-48 hours, after the break in the growth curve. After the onset of mass autolysis, nearly all of the vitamin is released from the mycelium into the medium.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

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# EFFECT OF $MnSO_4$ ON NATURE OF FERMENTATION BY Lactobactericum pentoaceticum IN DIFFERENT CONDITIONS OF AERATION AND ACIDITY OF THE MEDIUM

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Lactic acid bacteria, particularly the rod-shaped forms, are very fastidious as regards substrate.

The majority of lactic acid bacteria develop well only in such natural media as milk or wort. Only a few representatives can grow in media containing inorganic nitrogen. The vast majority of lactic acid bacteria require the presence of certain vitamins in the media. The mineral nutritional requirements of lactic acid bacteria have received little study. This is due to the fact that mineral elements are usually required in the small amounts present in organic substrates and even in pure chemical reagents. Hence, in the cultivations of lactic acid bacteria in complex natural media the question of adding mineral elements to them has usually not arisen. However, special investiagions (Wolley [1941]; MacLeod and Snell [1947, 1948, 1950]; Svec [1948]; and Evans and Niven [1951]) showed that the addition of the ions $K^+$, $Mg^{++}$, $Co^{++}$, $Fe^{++}$, $PO_4^{3-}$, etc., to relatively complex media containing protein hydrolyzates, vitamin sources, etc., shortened the lag phase, greatly increased the cell harvest and acid production in cultures of a number of lactic acid bacteria. Of all the elements, $Mn^{++}$ would appear to be of particular significance for lactic acid bacteria. A pronounced stimulating effect of $Mn^{++}$ on cell multiplication has been noted by many authors. The amount of $Mn^{++}$ necessary for maximal growth of the bacteria was found to be unusually high – 20-24 mg $Mn^{++}$ per liter of medium (Evans and Niven [1951]). The high sensitivity to manganese led to the development of a microbiological method of assaying $Mn^{++}$ by means of Lactobacillus arabinosum. The mode of action of manganese is still unclarified. Evans and Niven [1951] suggest that the effect of $Mn^{++}$ is connected in some way with the utilization of certain constituents of the medium, e.g., citrate. Leonard, et al. [1958], working with Bacillus subtilis, found that $Mn^{++}$ stimulated the synthesis of glutamic acid. The literature, however, contains no indications of the extent to which the effect of $Mn^{++}$ is related to other conditions of cultivation of the bacteria, such as the pH of the medium, aeration, etc., or if the addition of $Mn^{++}$ alters the proportions of the fermentation products.

In this investigation we studied the effect of $MnSO_4$ on the development and proportions of the products of the heterofermentative lactic acid fermentation by Lactobacillus pentoaceticum, isolated from kvass wort, in different conditons of aeration and acidity.

## METHOD

In our work we used 5.5° B* wort. The $MnSO_4$ was added in a dose of 110 mg per liter. The inoculum for the experiments in which the formed acids were neutralized consisted of a day-old culture from wort containing grains and chalk. For the media without neutralization the culture was passed through the experimental medium before being used for inoculation in the experiment. The inoculum comprised 1% of the culture. The temperature of cultivation was 37C.

The harvest of bacteria was estimated by a direct cell count on fixed preparations. The morphological changes in the cells were recorded by means of a drawing apparatus.

*Balling scale – Publisher.

Effect of $MnSO_4$ on Development of L. pentoaceticum in Wort of Different Strength, Without Chalk*

| Media | pH | | Cells per ml of medium, in mill | |
|---|---|---|---|---|
| | initial | final | expt. 1 | expt. 2 |
| 2° B wort without $MnSO_4$ | 6.21 | 3.28 | 202.71 | 189.5 |
| with $MnSO_4$ | 6.14 | 3.42 | 217.79 | 303.34 |
| 6° B wort without $MnSO_4$ | 5.96 | 3.26 | 450.66 | 490.68 |
| with $MnSO_4$ | 5.79 | 3.29 | 1129.26 | 953.52 |
| 11° B wort without $MnSO_4$ | 5.44 | 3.24 | 805.62 | 798.66 |
| with $MnSO_4$ | 5.18 | 3.12 | 1214.52 | 1508.58 |

* Duration of experiment—48 hr; initial number of bacteria—18.6 mill per ml.

The lactic acid was determined by Friedemann's method after the wort had been freed of proteins and carbohydrates. The acetic acid was determined by distillation with steam and $H_3PO_4$ and subsequent titration of the distillate with 0.1 N alkali. The lactic acid in the distillate was determined. Its amount was subtracted from the total acid content of the distillate. The remainder was expressed as acetic acid.

A paper chromatographic analysis showed that no other acids apart from lactic and acetic were formed in the wort during fermentation.

Ethyl alcohol was determined by Marten's method and by pycnometer.

Carbon dioxide in the experiments without neutralization was determined by displacing it from the acid wort by compressed, $CO_2$-free air into 0.1 N $Ba(OH)_2$ and subsequent titration of the baryta with 0.1 N oxalic acid.

Sugar was assayed by Bierry's micromethod after hydrolysis of the di- and polysaccharides. A special test showed that in wort without hydrolysis the sugar was not determined completely. Sugar was expressed as millimoles of trioses. All the products were expressed in millimoles per liter; the pH was measured electrometrically.

We give the mean data of two or several parallel experiments, with the control determinations in the sterile wort subtracted. Each experiment was conducted in two replicates.

We first tested the ability of L. pentoaceticum to grow in media commonly used for the cultivation of lactic acid bacteria. The following media were tested:

1. 2, 4, 6 and 10° B wort.

2. Wort + reducing agents (0.1 and 0.2% ascorbic acid and 0.01% hydrosulfite). According to Rabotnova [1957], Betabacterium pentoaceticum develops a little better in more reduced conditions. For this reason we used the reducing agents.

3. Media with vegetable extracts; the extracts were prepared by Shaposhnikov's method [1948] (in %): bean or soya extract—3, glucose—1, $K_2HPO_4$ + $KH_2PO_4$—0.05.

4. Medium containing peptone (in %): peptone—1; glucose—1; $KH_2PO_4$—0.05; $K_2HPO_4$—0.05; $MgSO_4$—0.02.

5. Medium containing mineral nitrogen (in %): glucose—1; $K_2HPO_4$—0.05; $KH_2PO_4$—0.05; $MgSO_4$—0.02; $(NH_4)_2SO_4$—0.1; yeast autolyzate—1.

The experiments were conducted in test tubes containing 20 ml of medium and no chalk. A comparison of the results showed that L. pantoaceticum was very exacting as regards substrates. Only 6-10° B wort secured the best development of the bacteria. Hence, in all our further work we used only wort.

## RESULTS

1. Effect of $MnSO_4$ on Harvest of L. pentoaceticum in Wort of Different Concentrations. The effect of $MnSO_4$ on the development of L. pentoaceticum was studied in conditions of limited access of air—in test tubes with 35 ml of medium (depth of medium—12 cm) on 2, 6, and 11° B wort without chalk. The results given in

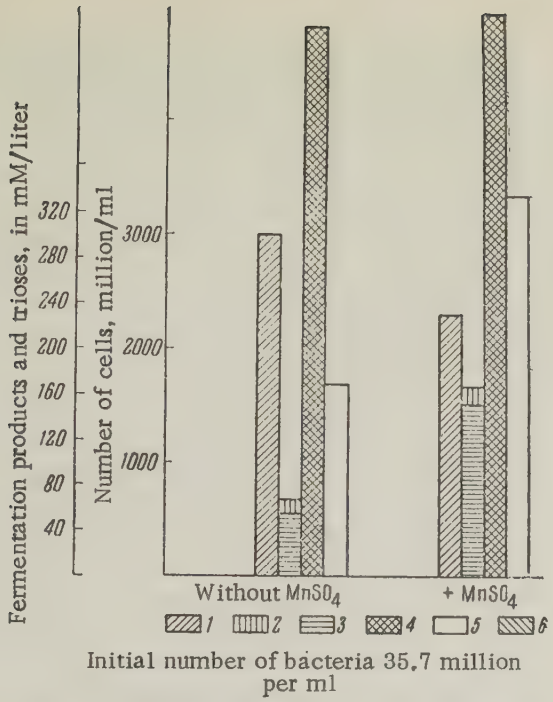


Fig. 1. Proportions of products of fermentation at optimal acidity (wort containing chalk) with limited access of air, with and without $MnSO_4$ (48 hr). 1) Lactic acid; 2) acetic acid; 3) alcohol; 4) sugar as trioses; 5) cells; 6) $CO_2$.

the table show that $MnSO_4$ very strongly stimulated the development of the bacteria, especially on the complete nutrient medium. In the diluted 2° B wort, where the development was inhibited by the lack of nutrient substances, the effect of $MnSO_4$ was much less pronounced The strongly stimulating effect of manganese on the bacterial multiplication led us to make a study of the effect of this compound on the nature of the fermentation by L. pentoaceticum. In the following work we used 5.5°B wort.

2. Effect of $MnSO_4$ on Development and Proportions of Fermentation Products of L. pentoaceticum in Conditions of Limited Access of Air. L. pentoaceticum is a facultative anaerobe. It develops much better with limited access of air (in test tubes) than with free access of air in flasks with a shallow layer of medium. When the bacteria develop in wort the medium is rapidly and strongly acidified to pH = 3.2-3.4. Owing to the excessive acidity the development of the culture ceases before the nutrient substances are exhausted. A good development and fuller utilization of the substrate are achieved when chalk is added to the medium to hold the pH of the wort at about 5, which is quite congenial for L. pentoaceticum.

a) Effect of $MnSO_4$ in wort with chalk (Fig. 1). In wort without $MnSO_4$ the maximal cell harvest was very high—more than 1.5 billion per liter, and the total yield of products was also very high. The main product was lactic acid. The lactic acid/alcohol ratio was 7/1. Acetic acid was produced in very small amounts. $MnSO_4$ doubled the harvest of bacteria but the productivity of each cell in the medium containing $MnSO_4$ was lower than in the medium without $MnSO_4$, since the total product yield and the sugar consumption were increased to a lesser extent. Thus, though $MnSO_4$ only slightly increased the total yield of fermentation products, it greatly stimulated cell division. This was confirmed by the change in their morphology: In wort containing $MnSO_4$ the cells were smaller and shorter than in wort without $MnSO_4$. In addition, $MnSO_4$ greatly altered the proportions of the fermentation products—the lactic acid yield was reduced and the alcohol yield was trebled. The lactic acid/alcohol ratio became 1.4/1.

b) Effect of $MnSO_4$ in naturally acidified wort. In fermentation in wort without neutralization (initial pH = 6.0) very acid conditions were created within 6-10 hr (ph = 3.2-3.4). The high acidity was uncongenial for the bacteria (Fig. 2). In wort without chalk the bacterial harvest was only slightly more than half of that in wort containing chalk, the quantity of fermentation products was appreciably reduced, and their proportions considerably altered. The lactic acid yield was most reduced, the yield of the neutral product—alcohol—was depressed relatively little, so that its relative amount was much higher than in fermentation with chalk. The lactic acid per alcohol ratio was 1/1. For each mM of hexose consumed there was almost 1 mM each of lactic acid, alcohol and $CO_2$. In these conditions $MnSO_4$ greatly stimulated the division of the bacteria (the bacterial harvest was 1.5 times higher, and the cells themselves were slightly smaller than in wort without $MnSO_4$). The quantity of sugar consumed was increased, the yield of fermentation products and their proportions were altered (Fig. 2). The yield of alcohol and $CO_2$ increased most appreciably. The lactic acid/alcohol ratio was 2/3 (sometimes 1/2). There was about 1.2 mM of alcohol and only 0.75 mM of lactic acid for each mM of hexose consumed. Thus, in conditions of limited access of air, irrespective of the pH, the addition of $MnSO_4$ to the wort stimulated the multiplication of the bacteria and greatly increased the yield of ethyl alcohol.

3. Effect of $MnSO_4$ on Development and Proportions of Products of Fermentation by L. pentoaceticum in Aerobic Conditions. Aerobic conditions were created by cultivating L. pentoaceticum in Winogradskii flasks containing 35 ml of medium, the depth of the medium being 1.5 cm.

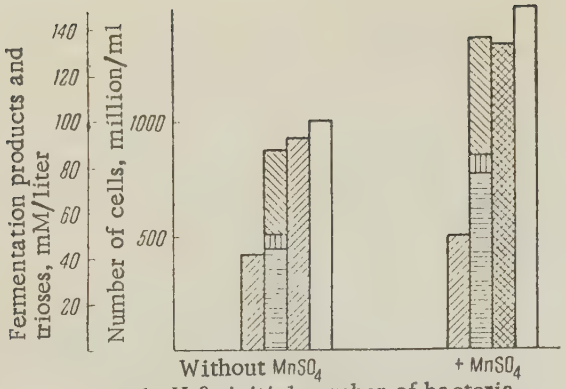
Fermentation products and trioses, mM/liter
Number of cells, million/ml
Without MnSO4
+ MnSO4
Initial pH 6; initial number of bacteria 43.5 million/ml

Fig. 2. Proportions of fermentation products in strongly acid wort in conditions of limited access of air, with and without $MnSO_4$ (46 hr). Conventions in Figs. 2-4 as in Fig. 1. Scale twice as large.

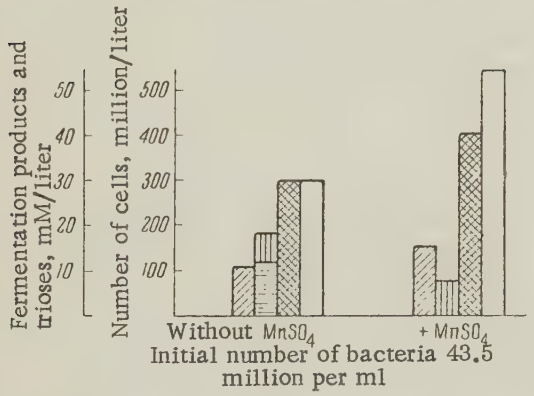
Fermentation products and trioses, mM/liter
Number of cells, million/liter
Without MnSO4
+ MnSO4
Initial number of bacteria 43.5 million per ml

Fig. 3. Proportions of fermentation products in wort containing chalk with aeration, with and without $MnSO_4$ (46 hr).

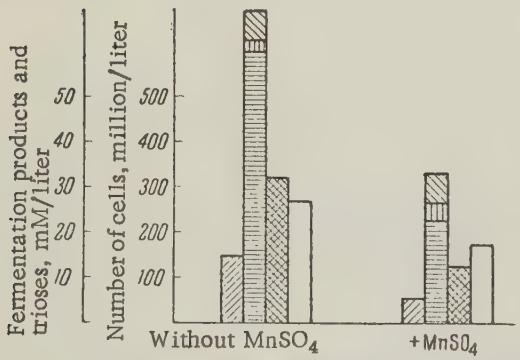
Fermentation products and trioses, mM/liter
Number of cells, million/liter
Without MnSO4
+MnSO4
Initial number of bacteria 43.5 million per ml

Fig. 4. Proportions of fermentation products in strongly acid wort with aeration, with and without $MnSO_4$ (46 hr).

a) Effect of $MnSO_4$ in wort containing chalk (pH about 5.0) (Fig. 3). Even in wort containing chalk, aeration greatly suppressed the formation of lactic acid and alcohol and increased the relative yield of acetic acid. Whereas it constituted an insignificant proportion in comparison with lactic acid in conditions of limited access of air, in aerated conditions the acetic acid production was 50% of that of the lactic acid. $MnSO_4$ stimulated bacterial division in these conditions: The cell harvest was almost twice as high, and the cells themselves smaller, than without $MnSO_4$. However, since the quantity of fermentation products did not increase in proportion to the number of cells, the productivity of each cell was reduced. A slight increase in sugar consumption gave an increase in the acid yield. However, the proportions of the acids were not affected. Twice as much lactic acid as acetic acid was produced. It must be pointed out that in aerated conditions $MnSO_4$ increased the absolute yield of acetic acid more appreciably than when access of air was limited. There was no formation of the reduced substance—alcohol. Thus, in the presence of $MnSO_4$ there was a more pronounced shift toward oxidative processes, and the effect of aeration was more marked than without $MnSO_4$. This is probably due to the more active participation of oxygen in the metabolism. It may be surmised that $MnSO_4$ takes some part in oxygen transfer. Possibly manganese is contained in enzymes catalyzing oxidation—reduction transformations.

As in the case of limited access of air, $MnSO_4$ altered the proportions of the fermentation products in the case of aeration in wort containing chalk, but in these conditions the nature of the change was quite different.

b) Effect of $MnSO_4$ in naturally acidified wort (Fig. 4). In aerobic conditions without chalk the development of bacteria was very weak owing to the simultaneous action of two adverse factors—oxygen and strong acidification. In these conditions L. pentoaceticum formed large amounts of the neutral product—alcohol. The alcohol was probably formed not only from carbohydrates but also from other substances in the wort. The lactic acid/alcohol ratio was 1/4 or 1/5. In these conditions $MnSO_4$ not only failed to stimulate, but even slightly lowered, the bacterial harvest. Apparently $MnSO_4$ checked the division of the bacteria, since in its presence the bulk of the cells were very elongated. $MnSO_4$ reduced the total yield of fermentation products, this being due to the reduction in the lactic acid and alcohol yields. However, the lactic acid/alcohol ratio remained as before – 1/4-1/5. The $CO_2$ yield was not reduced and the relative amount of this oxidized product was increased. Thus, in this case also, the addition of $MnSO_4$ catalyzed oxidative processes.

## DISCUSSION OF RESULTS

The effect of $MnSO_4$ on the development and nature of fermentation by L. pentoaceticum depends on the combination of several conditions of cultivation. When access of air to a complete nutrient medium (5.5°B wort) is limited, $MnSO_4$ stimulates bacterial multiplication, increases the yield of fermentation products, and alters their proportions. The general nature of the change in metabolism under the action of $MnSO_4$ is almost independent of the acidity of the medium. In strongly acid wort and in wort containing chalk, $MnSO_4$ slightly alters the lactic acid yield, hardly affects the acetic acid yield, and very greatly increases the alcohol production. As a result the lactic acid/alcohol ratio under the action of manganese changes from 7/1 to 2/3. Thus, in conditions of limited access of oxygen, $MnSO_4$ clearly emerges as an independent factor determining the proportions of the fermentation products of L. pentoaceticum. In aerobic conditions a different picture is observed. $MnSO_4$ enhances the effect of oxygen on the bacteria. It seems probable that manganese catalyzes oxidation processes and is possibly contained in oxidative enzymes. It acts in a different manner according to the acidity of the medium. At optimal acidity $MnSO_4$ stimulates multiplication of the bacteria and fermentation and promotes the participation of oxygen in the metabolism, thus preventing the formation of the reduced product—alcohol. In strongly acid conditions $MnSO_4$ checks bacterial division, reduces the total quantity of fermentation products, but does not reduce the $CO_2$ yield. This is also indicative of the stimulation of oxidation processes.

A comparison of the results shows that the metabolism of L. pentoaceticum can be greatly modified by a combination of cultivation conditions.

I express my deep thanks to Prof. I. L. Rabotnova for guidance in this work.

## SUMMARY

1. $MnSO_4$ greatly affects the multiplication and metabolism of Lactobacillus pentoaceticum in wort, but the nature of the effect of $MnSO_4$ depends on the combination of acidity and aeration conditions.

2. In a complete nutrient medium (5.5° B wort) with limited access of air, $MnSO_4$, irrespective of the acidity of the medium, greatly stimulates bacterial multiplication, increases the yield of fermentation products, slightly alters the lactic acid yield, and greatly increases the alcohol yield. The lactic acid/alcohol ratio changes from 7/1 without $MnSO_4$ to 2/3 with $MnSO_4$.

3. In aerated conditions $MnSO_4$ enhances oxidation processes. In wort containing chalk $MnSO_4$ stimulates bacterial multiplication, slightly increases the total volume of fermentation products by an increase in the acid yield, and completely prevents the production of the reduced product—alcohol. In strongly acid conditions $MnSO_4$ aggravates the harmful effect of oxygen on L. pentoaceticum, suppresses cell division, and reduces the lactic acid and alcohol yields. The yield of the most highly oxidized product ($CO_2$) is not reduced in this case.

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# NITRIFYING ACTIVITY OF PURE AND ENRICHED CULTURES OF *Nitrosomonas* ISOLATED FROM DIFFERENT NATURAL SUBSTRATES

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Studies of the nitrifying activity of enriched and pure Nitrosomonas cultures have revealed several characteristic features which distinguish pure cultures from the enriched cultures from which they were obtained. These features were inherent in all pure cultures of Nitrosomonas, irrespective of the natural substrates (different soils, manure, activated sludge) from which they were isolated. First of all there was established a regular and pronounced reduction of the nitrifying activity of Nitrosomonas cultures with increasing purification and isolation of pure cultures from them.

TABLE 1

Comparative Activity of Enriched and Pure Cultures in Different Nutrient Media (quantity of $NO_2$ in mg/liter after 10 days of experiment)

| No. of culture | Enriched cultures | | | | | Pure cultures | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | I | II | III | IV | V |
| 24a | 1989.7 | 2010.4 | 1997.5 | 2117.3 | 1996.6 | 18.4 | 18.9 | 18.87 | 18.6 | 29.7 |
| 73b | 974.5 | 981.4 | 996.7 | 1100.0 | 983.5 | 18.7 | 18.8 | 18.7 | 18.4 | 29.2 |
| 15s | 824.3 | 826.4 | 832.7 | 844.4 | 839.8 | 17.9 | 18.3 | 17.4 | 17.6 | 18.2 |
| 6c | 623.1 | 702.4 | 699.7 | 724.5 | 714.5 | 15.5 | 15.9 | 16.1 | 14.8 | 17.3 |
| 102a | 560.1 | 570.0 | 569.2 | 578.3 | 565.4 | 12.5 | 12.9 | 13.7 | 13.4 | 14.9 |
| 18s | 261.4 | 269.7 | 267.5 | 274.5 | 267.5 | 7.3 | 7.9 | 7.7 | 7.5 | 9, |

Note: I—Winogradsky's medium; II—Meiklejohn's medium B; III—Boltjes' medium; IV—medium containing $NH_4MgPO_4$; V—Winogradsky's medium + 1 ml 0.1% solution of peat humates.

The data of Table 1 show that the quantity of nitrites formed by pure Nitrosomonas cultures when grown in different mineral liquid media at 24-27 C was less by a factor 30-100 than that produced by the enriched cultures from which they were isolated. This feature of pure cultures has been noted by other investigators (Nelson [1931]; Imshenetskii and Ruban [1952, 1953]; Imshenetskii [1955]; Ruban [1955]).

In addition to other features (external form, absence of growth on organic nutrient media), this reduction in nitrifying activity can serve as one of the distinguishing characters of the purity of isolated Nitrosomonas cultures.

TABLE 2

Activity of Enriched and Pure Nitrosomonas Cultures in Successive Subcultures in Winogradsky's Medium at Ten-Day Intervals (quantity of $NO_2$ in mg/ liter)

| No. of culture | Enriched culture | | | | | Pure culture | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | initial activity | subculture | | | | initial activity | subculture | | | |
| | | 1 | 2 | 3 | 4 | | 1 | 2 | 3 | 4 |
| 32a | 1715.6 | 1713.9 | 1714.7 | 1712.8 | 1710.9 | 8.92 | 8.74 | 7.39 | 6.45 | 6.54 |
| 17s | 618.4 | 615.6 | 616.7 | 613.4 | 614.8 | 7.1 | 6.32 | 6.15 | 5.85 | 4.98 |
| 21c | 566.3 | 563.7 | 562.4 | 564.5 | 561.4 | 6.2 | 5.75 | 5.17 | 4.94 | 4.11 |
| 19c | 341.5 | 339.4 | 337.6 | 338.9 | 336.7 | 4.81 | 4.24 | 4.07 | 3.79 | 3.54 |
| 114s | 263.1 | 260.8 | 261.4 | 260.9 | 260.4 | 3.42 | 2.69 | 2.12 | 1.97 | 1.14 |

The nitrifying activity of the Nitrosomonas cultures was determined colormetrically from the nitrite accumulation and ammonia consumption by means of the Griess and Nessler reagents.

In the experiments on the quantitative determination of ammonia and nitrites in Nitrosomonas cultures in various mineral liquid media (especially in the study of the dynamics of the processes in these media) the control consisted of flasks containing sterile media.

The enriched and pure Nitrosomonas cultures were grown in the liquid mineral media indicated in our previous work (Ul'yanova [1960]).

A positive reaction for nitrites and the maximum accumulation of them always occurred much later in pure Nitrosomonas cultures than in the corresponding enriched cultures.

Whereas the greatest quantity of nitrites in the liquid media was formed by enriched cultures after 15-20 days of cultivation at room temperature, the greatest accumulation of nitrites in the corresponding pure cultures occurred 10-15 days later, irrespective of which enrichment culture they had been obtained from. Similar results were also obtained in the cultivation of Nitrosomonas cultures on silica gel. The number of zones of solution of the chalk or $NH_4MgPO_4$ in pure Nitrosomonas cultures was only a fifth to a third of the number in the corresponding enriched cultures. The diameter of the zone was usually less than 0.1 cm, whereas the mean diameter of the zone of "clearing" in the enriched cultures reached 0.5-1 cm. It was later found that with successive subcultures of pure Nitrosomonas cultures in liquid mineral media there was a gradual, and sometimes even sharp, reduction in their initial nitrifying activity (Table 2).

During the work this often led to the death of the pure Nitrosomonas cultures and the need to isolate them again from enriched cultures.

The enriched Nitrosomonas cultures, on the other hand, from which pure cultures were repeatedly isolated, remained in excellent condition throughout the time of the investigations (more than 2.5 yr). They did not require frequent subculturing and did not lose their activity. This ephemeral existence of pure Nitrosomonas cultures and the reduction of their activity with successive subculturing is difficult to explain at present. The only clear point is that in the conditions of enriched cultures Nitrosomonas is able to oxidize ammonia more rapidly. We can deduce from this that the concomitant bacteria have a favorable effect on the development of the nitrite bacteria. This fact has been pointed out by several investigators who have worked with this group of bacteria (Stapp [1940]; Nechaeva [1947]; Mekhtieva [1954]; Imshenetskii [1955]; Ruban [1955]).

When the concomitant bacteria are present the activity of Nitrosomonas is greatly enhanced. For instance, according to the data of Imshenetskii and Ruban [1953], a mixed culture of nitrite bacteria formed 123 mg $NO_2$ per liter in 20 days, whereas a pure culture formed only 10 mg $NO_2$ per liter.

The mode of action of the Nitrosomonas associates has not yet been clarified. It is possible that they may modify the oxidation–reduction potential of the medium or consume the metabolic products of Nitrosomonas, thus enabling it to develop better. Only the addition of a 0.1% solution of peat humates to the liquid mineral media slightly checked the loss of activity in pure Nitrosomonas cultures, and in some cases even increased their activity (by a factor 1.5-2 in comparison with liquid mineral media, Tables 1 and 3).

A study of the nitrifying activity of enriched and pure cultures of Nitrosomonas isolated from different

TABLE 3

Comparative Activity of Enriched and Pure Nitrosomonas Cultures Isolated from Various Natural Substrates (quantity of $NO_2$ in mg/liter after 15 days of experiments)

| Substrates from which culture were isolated | Enriched cultures | | | Pure cultures | | | |
|---|---|---|---|---|---|---|---|
| | I | II | III | I | II | III | IV |
| Activated sludge from aerated tanks | 1996.5 | 2021.2 | 2125.3 | 19.1 | 19.3 | 18.9 | 19.9 |
| Cow manure | 986.3 | 985.9 | 1000.9 | 17.4 | 17.6 | 18.0 | 18.4 |
| Horse manure | 876.0 | 880.6 | 889.3 | 15.5 | 15.6 | 14.7 | 16.2 |
| Chernozem from Kamennaya Steppe | 864.1 | 870.3 | 898.7 | 19.5 | 19.7 | 19.2 | 19.8 |
| Chernozem from Bulgaria | 673.6 | 668.7 | 689.0 | 16.9 | 17.1 | 17.2 | 17.3 |
| Chernozem from Tellerman Experimental Forestry | 521.9 | 529.8 | 570.3 | 15.7 | 15.7 | 14.7 | 15.9 |
| Solonetz from Kamennaya Steppe | 425.1 | 417.4 | 429.9 | 12.2 | 13.3 | 12.5 | 16.0 |
| Solonetz from Tellerman Experimental Forestry | 304.1 | 307.6 | 329.3 | 11.3 | 11.5 | 12.2 | 14.9 |
| Dark grey forest soil from Tellerman Experimental Forestry | 475.0 | 491.3 | 501.4 | 15.1 | 15.3 | 14.2 | 16.3 |
| Sandy soil from south Karelia | 324.2 | 328.6 | 340.4 | 13.1 | 12.6 | 12.5 | 14.2 |
| Sandy soil from Tellerman Experimental Forestry | 287.4 | 290.1 | 299.9 | 12.4 | 13.7 | 12.8 | 14.3 |
| Alpine soil from Bulgaria | 243.6 | 240.5 | 249.4 | 7.6 | 8.4 | 7.8 | 9.2 |

Note: I—Winogradsky's medium; II—Meiklejohn's medium containing microelements; III—medium containing $NH_4MgPO_4$; IV—Winogradsky's medium + 1 ml 0.1% solution of peat humates.

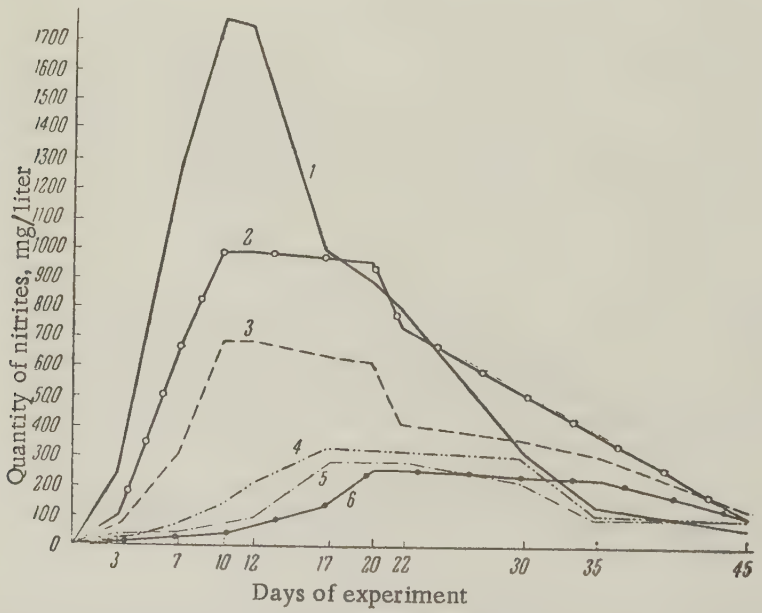


Fig. 1. Dynamics of nitrite formation by various enriched Nitrosomonas cultures in Winogradsky's medium. 1) Culture from activated sludge; 2) culture from Kamennaya Steppe chernozem; 3) culture from Bulgarian chernozem; 4) culture from sandy soil of south Karelia; 5) culture from Tellerman sandy soil; 6) culture from Bulgarian alpine soil.

natural substrates showed that with identical conditions of culturing in nutrient media of the same type inoculated with approximately the same number of cells there were differences in the activity of the cultures. This can be seen from the data in Table 3.

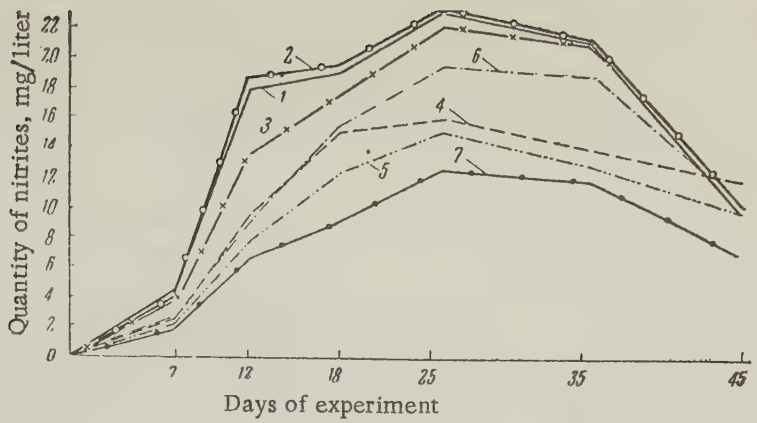


Fig. 2. Dynamics of nitrite formation by various pure Nitrosomonas cultures in Winogradsky's medium. 1) Culture from activated sludge; 2) culture from Kamennaya Steppe chernozem; 3) culture from cow manure; 4) culture from Kamennaya Steppe solonetz; 5) culture from sandy soil of south Karelia; 6) culture from dark grey forest soil of Tellerman; 7) culture from Bulgarian alpine soil.

The most active of the enriched Nitrosomonas cultures were those obtained from activated sludge, manures and the KamennayaSteppe chernozem, while cultures isolated from the two other chernozems, the dark grey forest soil, the solonetz and sandy soils were less active. The Nitrosomonas cultures isolated from Bulgarian alpine soil had the lowest nitrifying activity.

It may be noted that a positive reaction for nitrites appeared two to five days earlier in the more active enriched cultures, and the accumulation of the greatest quantity of nitrites (10-12th day of experiment) occurred three to ten days earlier than in less active cultures, irrespective of the type of mineral liquid medium (Fig. 1). The process of ammonia oxidation terminated 8-14 days earlier in the more active Nitrosomonas cultures than in the less active cultures.

As regards the pure Nitrosomonas cultures the greatest quantity of nitrites was formed in cultures isolated from activated sludge and the Kamennaya Steppe chernozem. These were followed by cultures obtained from the Bulgarian chernozem, cow manure, chernozem from Tellerman Experimental Forestry, dark grey forest soil, horse manure, solonetz and sandy soils and, lastly, cultures from alpine soil (Table 3.)

The pure cultures of different origin differed markedly in activity. A comparison of the times of appearance of the positive reaction for nitrites, their formation in maximum quantity, and the rate of ammonia oxidation in pure Nitrosomonas cultures did not show the differences typical of the enriched cultures. In almost all the cultures a positive reaction for nitrites appeared on the 6th-10th day of the experiment, the greatest quantity of nitrites was formed by the 20th-27th day, and the oxidation of ammonia was completed on the 30th-36th day of the experiment (Fig. 2).

The activity of the investigated enriched and pure Nitrosomonas cultures depended on the specific features of the natural substrates from which these cultures were obtained. In substrates rich in organic matter—in our experiments the activated sludge (total carbon 43.39% of dry weight of mud), manure, chernozems from south Bulgaria, Tellerman Experimental Forestry, and Kamennaya Steppe (14.36%, 9.82%, and 8.26% humus, respectively ), dark grey forest soil (12.20% humus), and Kamennaya Steppe solonetz (8.16-7.53% humus)—the processes of mineralization of organic matter are more rapid (intense ammonification and other processes). Nitrosomonas strains exhibiting great activity in oxidizing ammonia were adapated to such substrates.

In media with a lower organic matter content—sand soils of Karelia and Tellerman Experimental Forestry (6.15% and 2% humus, respectively), solonetz from Tellerman Experimental Forestry (3.88% humus)—the course of biochemical processes is evidently slower and, in consequence, Nitrosomonas strains possessing a low nitrifying activity develop in them (for a chemical characterization of the samples of natural substrates, see Ul'yanov [1960]).

The Nitrosomonas strains inhabiting the alpine soil of Bulgaria (20.42% humus) showed a particularly low nitrifying activity.

Similar facts have been reported in the papers of other investigators who have studied nitrification in various substrates. For instance, in the tundra soils of Yamal Pen. Severin [1909] detected nitrification only in one out of six investigated samples, the one taken from garden soil in Obdorsk. He related this phenomenon to the amount of organic matter in soils of this type.

The nitrification process is extremely weak in nothern sandy soils containing a very small amount of organic matter (1.9-2.1%) and with high acidity (pH 3.5-3.7) (Tsyplenkin and Zhilin [1936]).

According to the results of several authors, sandy soils also have a low nitrifying ability. The sandy soils of the R. Desna terrace, which are situated on ancient alluvial sands in pine groves with humus (1-2%), and which have an acid reaction (pH 4.5-5.5) and low content of bases (10-20%), are distinguished by the complete absence or weak manifestation of nitrification (Remezov [1951]). A suppression of the nitrification process in sandy and loamy soils of the oak groves of the Saratov-Astrakhan shelterbelt was noted by Glotova [1950].

We know that only soils in which the processes of decomposition of organic matter are rapid and which contain large number of ammonifiers (Bacillus megaterium, Bacillus mesentericus, Bacillus idosus, etc.) display intense nitrification (Mishustin and Pertsovskaya [1954]; Mitrofanova [1953]; Pushkinskaya [1954]; Korsakova [1930]; Kostychev [1930]; Kolcheva [1954]).

Dark-colored alpine soil, according to Kolcheva's investigations, is characterized by very weak biogenicity calculated per 1 g of soil and per unit dry weight of humus. The low content of ammonifiers in it is the cause of the "dry peat formation" and the associated high humus content (20%). This is also promoted by the damp and cold climate of alpine regions. These are relatively young soils, which lack the sufficiently well formed organic material specific for the soil type. The extreme poverty of sporing forms of microorganisms is a consequence of their age, since genetically young soils always contain very small numbers of sporing forms of microorganisms.

The factual information which we obtained in a study of the nitrifying activity of pure and enriched Nitrosomonas cultures shows that these bacteria actually adapt themselves to the conditions of the environment in which they live. In view of this an ecological approach to the study of the agents of the first phase of nitrification is the only way of explaining the differences in the activity of cultures, a feature noted by several investigators but not explained by them on the basis of the unity of the organism and environment.

## SUMMARY

1. A study of the nitrifying activity of pure and enriched Nitrosomonas cultures showed that enriched cultures exhibited much greater activity (30-100 times greater) than pure cultures isolated from them. This difference in activity was probably due to the favorable effect of concomitant bacteria on the Nitrosomonads.

2. Successive subcultures of pure cultures showed a reduction in activity and the cultures gradually died off in laboratory conditions. This was evidently due to the fact that the optimal conditions for cultivation of this group of bacteria have not yet been discovered.

3. Nitrosomonas strains isolated from different natural substrates exhibit different nitrifying activity. The more intense the microbiological processes in the substrate (ammonification, etc.), the greater is the nitrifying activity of Nitrosomonas cultures isolated from these substrates. This is indicative of the ecological adaptation of Nitrosomonas to their conditions of existence.

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# MECHANISM OF ACTION OF LEVOMYCETIN (CHLORAMPHENICOL)

## V. EFFECT OF LEVOMYCETIN ON PYRUVATE CONSUMPTION BY RESTING CELLS OF Escherichia coli (Bacterium coli)

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In a preceding report (Korotyaev, 1959) it was shown by us that the utilization by resting cells of Escherichia coli (Bacterium coli) takes place by varying pathways depending on the degree of aeration of the medium. At the same time it was established that chloramphenicol has no significant effect on the aerobic utilization of pyruvic acid, but sharply inhibits the anaerobic pathway of assimilating pyruvate.

Among anaerobic organisms, and among aerobic microorganisms under anaerobic conditions, the utilization of pyruvate can follow various pathways with the participation of different enzyme systems. Fungal cells which have carboxylase convert pyruvic acid into acetaldehyde and $CO_2$. The final product of the conversion of glucose by lactobacilli is lactic acid. Furthermore, pyruvic acid can be converted into acetylmethylcarbinol (acetoin). This pathway of utilization of pyruvate has been established in Aerobacter aerogenes, Proteus morganii, and other bacteria. Several microorganisms oxidize acetaldehyde which is formed during the breakdown of pyruvate into acetic acid with the participation of the corresponding dehydrogenase. The enzyme mechanism found in a number of bacteria as well as in animal tissues which catalyzes the conversion of pyruvate into acetyl-coenzyme A is particularly significant. The activity of this system is tied up with the presence of such cofactors as thiamine pyrophosphate, coenzyme A, DPN, and also Mg ions.

Kalnitsky and Werkman (1943) showed that a cell-free preparation of E. coli under anaerobic conditions broke down pyruvate with the formation of acetic, formic, and lactic acids, and $CO_2$. The formation of acetic and formic acids from pyruvate was initially considered to be a hydrolytic reaction. Utter, Werkman, and Lipmann (1944) showed that it represented a phosphorylation reaction whose intermediate product was acetylphosphate. This phosphorylation reaction is reversible. E. coli formed the adaptive enzyme hydrogenlyase which catalyzes the reversible breakdown of formic acid into $CO_2$ and molecular hydrogen. Cell-free extracts of Clostridium butylicum also catalyze the breakdown of pyruvate into acetic acid, $CO_2$ and molecular hydrogen, but during the reaction formic acid is not formed as an intermediate product (Koepsell and Johnson, 1942). The formation of acetylphosphate as an intermediate product in this reaction is understood although no one has succeeded in isolating it (Koepsell, Johnson and Meek, 1944). The mechanism of the phosphorolytic breakdown of pyruvate by E. coli is, up to the present, poorly understood. Phosphate, coenzyme A and cocarboxylase are necessary as cofactors for this system. However, the observations of Kalnitsky and Werkman have shown that cell-free preparations of E. coli after dialysis for 1.5 to 2 hours are inactivated and the activity cannot be restored upon the addition of phosphate, cocarboxylase and manganese. The further addition of coenzyme A, adenylic acid and ATP, magnesium, riboflavin, fumarate, calcium pantothenate or biotin in different combinations does not appreciably restore the activity to the dialyzed enzyme preparation, while the addition of boiled extract of fungi gives a positive result. It is possible that other components together with those enumerated are a part of this system.

As regards microorganisms it was of interest to study the paths of conversion of pyruvate among microorganisms in general and E. coli in particular in their utilization of pyruvate under those experimental conditions

which were used by us to study the effects of chloramphenicol on the metabolism of pyruvic acid in E. coli and to elucidate what reaction this antibiotic inhibits.

TABLE 1

The Effect of pH on the Speed of Pyruvate Utilization of Resting Cells of E. coli

| pH | 5.6 | 6.2 | 6.8 | 7.2 | 7.8 | 8.4 |
|---|---|---|---|---|---|---|
| Pyruvate utilization in 4 hr, μM | 27.0 | 30,6 | 32.4 | 17.4 | 14.4 | 11.4 |

## MATERIALS AND METHODS

The investigations were carried on with the same strain of E. coli that was used in our previous experiments. The bacteria were grown on the usual beef-peptone agar for 20-24 hr, after which they were washed with saline and centrifuged 5 times at 2500 rpm so that they were washed free of the nutritive medium. The suspension so obtained of resting cells was preserved in the cold. The effect of the antibiotic on the utilization of pyruvate by the bacteria under standard conditions was studied by the following method. Into two test tubes 1-1.5 cm in diameter were introduced 3 to 6 ml of 0.4% sodium pyruvate, 5 ml of phosphate buffer, 0.5 ml of antibiotic solution or 0.5 ml of 0.85% saline and 1 ml of a suspension of resting cells. The original concentration of pyruvate was determined and the test tubes were placed for several hours in a 37 C bath. Colorimetric determinations of the concentration of pyruvate were carried out with a photoelectric colorimeter FÉK-M according to a modification of the method of Freedman and Haudgen. Volatile acids were determined by distillation after the method of Duclos. Acetic acid was quantatively determined and formic acid was determined by the method of Grant (1948). For the determination of acetylphosphate the method of Lippman and Tuttle (1945) was used. The determination of lactic acid was carried out according to a modification of Barker and Samerson. The determination of the oxidation-reduction potential of the medium was carried out electrometrically with the aid of a lamp potentiometer LP-5 and platinum electrodes.*

TABLE 2

Relation between the Concentration of Levomycetin and the Degree of its Inhibitory Activity on the Utilization of Pyruvate by the Cells of E. coli

| Concentration of levomycetin, μg/ml medium | 0 | 1.0 | 2.0 | 7.8 | 31.0 | 62.0 | 125.0 |
|---|---|---|---|---|---|---|---|
| Pyruvate utilization in 4 hr, μM | 91.8 | 57.7 | 35.9 | 34.5 | 17.2 | 16.1 | 4.5 |
| % Inhibition | 0 | 37.2 | 61.9 | 63.5 | 81.3 | 82.8 | 95.0 |

## EXPERIMENTAL RESULTS

Our initial observations on studying the effect of chloramphenicol on the utilization of pyruvate by resting cells of E. coli were carried out at pH 7.2-7.4. Therefore, to elucidate the effect of pH on the speed of the reaction, the results which are shown in Table 1 were established.

As is seen in Table 1, the speed of the reaction was significantly greater in acid than in alkaline medium. The maximal reaction rate was observed at pH 6.8. In all the following experiments phosphate buffer at pH 6.8 was used.

*This portion of the investigation was carried out by the Department of Microbiology at the M. V. Lomonosov Moscow State University. The author wishes to thank L. A. Mineevaya for help in the determination of the oxidation-reduction potential.

The preparation of cocarboxylase was kindly presented to us by docent L. A. Malakhovsky (from Vilnus). The preparation of dextromycetin was kindly supplied by M. M. Shemyakiny.

TABLE 3

Effect of Dextromycetin on the Rate of Pyruvate Utilization of Resting Cells of E. coli

| Dextromycetin, μg/ml medium | 0 | 2.0 | 7.8 | 31.0 | 62.0 | 125.0 | 250.0 |
|---|---|---|---|---|---|---|---|
| Pyruvate utilization in 4 hr, μM | 58.8 | 60.3 | 58.8 | 55.1 | 47.3 | 35.7 | 24.1 |
| % Inhibition | 0 | 0 | 0 | 6.3 | 19.6 | 39.3 | 60.0 |

In Table 2 are given the results of one of the experiments which illustrates the dependence between the concentration of antibiotic and the degree of its inhibition on the utilization of pyruvate. As the concentration of chloramphenicol was increased,so the suppressive activity was increased. Thus the inhibition by the antibiotic of pyruvate utilization has a specific character.

In order to elucidate whether the inhibition of pyruvate utilization was a specific property of chloramphenicol, experiments were performed with an isomer of chloramphenicol, dextromycetin. As is well known, of the four isomers of chloramphenicol only the levomycetin form possesses high biological activity. The remaining three isomers are almost devoid of activity. The results of the experiment with dextromycetin [L-threo-(1-p-nitrophenyl)-2-dichloroacetylamido-1,3-propanediol] are given in Table 3.

From the data given in Tables 2 and 3 it is evident that dextromycetin is in many respects a weaker inhibitor of pyruvate utilization than levomycetin. A levomycetin concentration of 1 μg per ml inhibits the rate of pyruvate utilization by 37%, whereas dextromycetin requires a concentration of 125 μg per ml to attain the same inhibition. The inhibitory activity of dextromycetin on pyruvate utilization by resting E. coli is almost 100 times weaker than the activity of levomycetin. These data confirm the high degree of specificity of levomycetin activity and at the same time show that the ability of the antibiotic to suppress the process of pyruvic utilization is a reflection also of its biological activity.

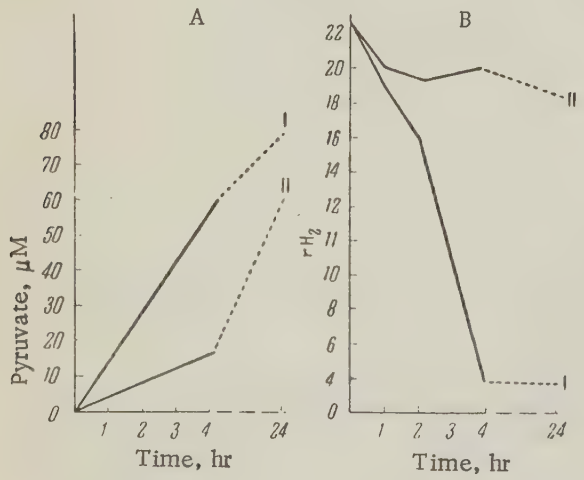


Fig. 1. Effect of levomycetin on the rate of pyruvate utilization (A) and changes in the oxidation-reduction potential (B) of resting cells of E. coli under standard conditions. I) Without antibiotics; II) in the presence of levomycetin (128 μg per ml).

A significant difference was established in the changes in oxidation-reduction potential in the medium during pyruvate utilization in resting E. coli cells under standard conditions. As seen in Fig. 1, under standard conditions in a medium without antibiotic during pyruvate utilization a sharp decline in potential occurs; $rH_2$ of the medium falls from 22.7 to 1.7 and remains at this level for 24 hr. In the presence of levomycetin only an insignificant drop in potential was observed in the course of the first two hours of the experiment and then the potential stayed constant for almost 24 hr. Such a difference in changes of $rH_2$ in the medium are apparently correlated with the formation of different end products of pyruvate breakdown,but not with different rate of its utilization. In the absence of levomycetin over 4 hr when utilization of 60 micromoles of pyruvate occurs the potential falls to the lowest level ($rH_2$ equals 1.7). The utilization of the same amount of pyruvate in the presence of the antibiotic over 24 hr does not bring about a significant lowering of the $rH_2$. Apparently under standard conditions the cells use pyruvate with the formation of some reduction products which do not accumulate in the presence of the antibiotic.

The results of the measurements of the oxidation-reduction potential are an indirect support for the conclusion reached by us earlier that under standard conditions the utilization of pyruvate by the resting cells of E. coli

follows anaerobic pathways. From Fig. 2B, it is seen that in pyruvate utilization under standard conditions of constant aeration of the medium, the potential of the medium remains constantly high toward the end of the experiment and when all the pyruvate is oxidized it goes up slightly. Levomycetin to a very slight extent inhibits the rate of pyruvate utilization under these conditions (A) and its presence is not reflected in the changes of potential.

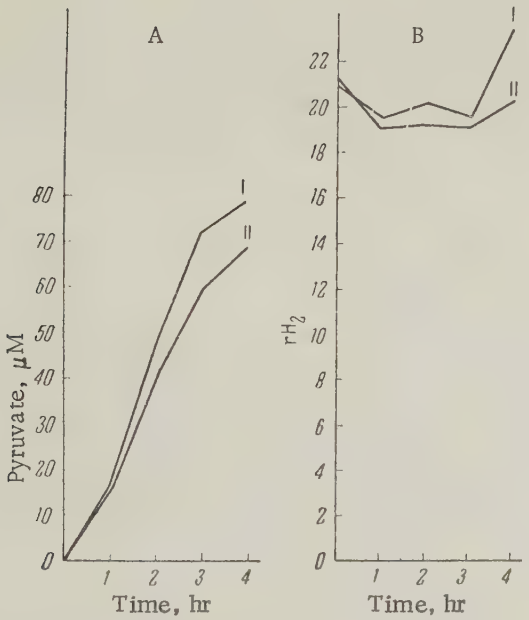


Fig. 2. Effect of levomycetin on the utilization of pyruvate (A) and changes in the oxidation-reduction potential (B) of resting cells of E. coli under aerobic conditions. Designations the same as in Fig. 1.

The sharp fall in $rH_2$ during pyruvate utilization by the resting cells under standard conditions indicates the accumulation of some reduction compounds, one of which may be lactic acid. Many observations have shown that lactic acid is indeed formed during this process, but in insignificant amounts, so that the sharp decline in $rH_2$ can hardly be explained in this way.

During the distillation of volatile acid only acetic and formic acids were qualitatively recovered. Quantative determinations were carried out on volatile acids. The pyruvic acid remaining in the medium was precipitated with 2,4-dinitrophenylhydrazine. In order to avoid any errors due to the presence of acetic acid or of other volatile acid products, the determination of the volatile acids was carried out after repeated distillation. Of course a portion of the volatile acid is lost, which must be taken into account in the analysis of the result obtained. As is seen in Table 4, the final products of pyruvate conversion were acetic, formic and lactic acids. In the absence of antibiotic the formation of gas was observed. The nature of the gas was not determined, but apparently it consists of $CO_2$ and molecular hydrogen. As the lactic acid which is formed is insignificant in amount, it must be considered that under standard conditions of the resting cells of E. coli pyruvate is quantitatively converted into acetic and formic acids.

$$CH_3COCOOH \rightarrow CH_3COOH + HCOOH$$

As has already been noted, it was considered that in the course of this reaction the intermediate product was acetylphosphate, which then was hydrolyzed to acetic acid. However, several of our experiments were designed to observe the formation of acetylphosphate during the conversion, but they did not succeed in bringing about positive results.

$$CH_3COCOOH \rightarrow CH_3COOPO_3H_2 + HCOOH$$

$$CH_3COOPO_3H_2 + H_2O \rightarrow CH_3COOH + H_3PO_4$$

It is possible that in pyruvate utilization the formation of acetylphosphate occurs so quickly that no accumulation of it occurs in the medium. Lactic acid is formed under these conditions indeed by dismutation. From Table 4 it is seen that in the presence of levomycetin formic and acetic acids are observed almost in equal proportions, while without the antibiotic significantly less formic acid is observed than acetic acid, the latter being formed in greater amount. It is apparent that formic acid is further converted under these circumstances into $CO_2$ and molecular hydrogen. Hydrogen given off during the conversion of formic acid is the cause of the sharp decline of the $rH_2$ during the breakdown of pyruvate under standard conditions by the resting cells. In the presence of levomycetin this reaction is completely inhibited. The formation of gas was not observed in a single experiment.

$$HCOOH \rightarrow CO_2 + H_2$$

TABLE 4

Effect of Levomycetin on the Formation of End Products of Pyruvate Utilization by Resting Cells of E. coli under Standard Conditions

| Content of levomycetin in the medium, μg per ml | Pyruvate utilization in 4 hr, μM | End products, μM | | | |
|---|---|---|---|---|---|
| | | $CH_3COOH$ | HCOOH | $CH_3CHOHCOOH$ | gas formation |
| 0 | 172 | 127 | 66 | 1.3 | ++ |
| 25 | 94 | 70 | 67 | 0.5 | — |
| 0 | 167 | 142 | 75 | 3.0 | ++ |
| 25 | 81 | 78 | 61 | 0.5 | — |

TABLE 5

Effect of Levomycetin on theFurther Breakdown of the End Product of Pyruvate Utilization by Cells of E. coli under Standard Conditions

| Original pyruvate content, μM | Levomycetin μg/ml | Gas formation | In 5 hr | | | In 12 hr | | | In 24 hr | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pyruvate utilization, μM | $CH_3COOH$, μM | HCOOH, μM | pyruvate utilization, μM | $CH_3COOH$, μM | HCOOH, μM | pyruvate utilization, μM | $CH_3COOH$, μM | HCOOH, μM |
| 184 | 0 | ++ | 173 | 110 | 54 | 178 | 126 | 14 | 180 | 130 | 1.3 |
| 184 | 25 | — | 78 | 54 | 36 | 129 | 84 | 56 | 166 | 92 | 88 |

In addition, as has already been noted above, in the presence of levomycetin pyruvate utilization by E. coli under standard conditions is not accompanied by a sharp fall in potential.

Thus, the investigations which were carried out showed that levomycetin sharply inhibits the phosphorylative breakdown reaction of pyruvic acid by E. coli. There remains, however, the need to clarify the mechanism by which this reaction is inhibited. As formic acid which is formed in the presence of levomycetin does not undergo further conversion, one might hypothesize that the inhibition of the reaction is a consequence of the accumulation of excessive amounts of end products; that is, in the presence of antibiotic a displacement of equilibrium toward pyruvate occurs. To explain the ability of such a mechanism to inhibit the phosphorylative reaction it is necessary to verify further the fate of the end product of pyruvate conversion. The results of this experiment, which are presented in Table 5, show conclusively that during pyruvate breakdown acetic acid is formed in the presence of antibiotic and without it and apparently does not undergo any further change. Therefore the accumulation of acetic acid cannot serve as an explanation for the inhibition of the reaction. The matter of formic acid is quite to the contrary. In the presence of levomycetin, the formic acid which is formed does undergo further conversion with the formation of a gas. In the presence of the antibiotic the breakdown of pyruvate by E. coli is accompanied by the accumulation of formic acid and the absence of gas formation. Evidently levomycetin almost completely suppresses the reaction of the further utilization of formic acid. Nonetheless, the accumulation of formic acid must not be considered as a cause of inhibition of the reaction of pyruvate breakdown. As is seen in Table 5, in the absence of levomycetin, over 5 hr almost all the pyruvate is assimilated and during this period 54 micromoles of formic acid are found; in the presence of levomycetin during 5 hr significantly less formic acid is found, but the rate of pyruvate utilization is inhibited more than two times as much. Evidently the accumulation also must be considered as the cause of the inhibition of the reaction.

Understandably, the breakdown of formic acid is catalyzed by the enzyme hydrogenlyase. It would be of interest to explain if the antibiotic inhibits the syntheses of hydrogenlyase or destroys its activity. With this aim in mind the following experiments were performed. To a suspension of washed cells of E. coli in phosphate buffer was added sodium formate, and the cells were maintained for several days in an incubator. Gas formation

during this time was not noted and such cells did not contain hydrogenlyase. For the formation of hydrogenlyase, an adaptive enzyme, a suspension of cells was added to a liter of pyruvate solution in phosphate buffer and incubated for several days in the incubator. The cells were washed free of pyruvate by twice centrifuging them at 2500 rpm and were then tested in an experiment with sodium formate and sodium pyruvate. The presence of hydrogenlyase in this instance was established by the formation of gas in the medium with formate. It was realized that such cells which contain hydrogenlyase resulted in gas formation in the medium of pyruvate in the presence of antibiotic. Fig. 3 demonstrates the effect of antibiotic on the rate of pyruvate utilization by cells which contain adaptive enzyme and by those which do not. Levomycetin inhibits the activity of both to the same degree. Consequently, independent of whether hydrogenlyase is present or absent in the cells of E. coli, levomycetin sharply inhibits the reaction of pyruvate breakdown. In addition, the antibiotic suppresses the synthesis of adaptive enzyme which catalyzes the conversion of formic acid.

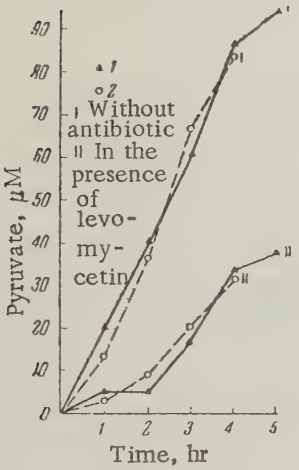


Fig. 3. Effect of levomycin on the rate of pyruvate utilization of resting cells of E. coli. 1) Cells containing adaptive enzyme (hydrogenlyase); 2) cells without adaptive enzymes.

## DISCUSSION

As other investigations have shown, levomycin selectively inhibits the reaction of pyruvate breakdown into acetic and formic acids. It is known that pyruvate is one of the most important sources of acetylated coenzyme A, either by means of formation of acetyl phosphate (Stadtman, Novelli, Lipmann, 1951), or of its indirect formation (Chantrenne, Lipmann, 1950; Chou, Lipmann, 1952); that in suppressing the process of pyruvate breakdown levomycin destroys at the same time the acetylation of coenzyme A. Further elucidation of the fact of levomycin inhibition in the reaction of pyruvate breakdown should shed light on the nature of one of the possible means of levomycin interference in the metabolism of the microbial cell.

In conclusion the author wishes to thank Professor V. N. Shaposhnikov and Professor Z. S. Gershenovich for their valuable advice and help in this work.

## SUMMARY

1. Under standard conditions the consumption of pyruvic acid by resting Bacterium coli under limited aeration of the medium results in its breakdown into acetic and formic acids with concomitant formation of a small amount of lactic acid.

2. In the course of pyruvate consumption the cells of the Bacterium coli synthesize an adaptive enzyme (hydrogenlyase) which catalyses further splitting of formic acid.

3. Levomycetin inhibits synthesis of the adaptive enzyme which catalyses splitting of formic acid.

4. Irrespective of inhibition of the adaptive enzyme synthesis, levomycetin inhibits pyruvate breakdown by the cells of the Bacterium coli into acetic and formic acids.

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# PROLONGED RETENTION BY BACTERIA OF THE ABILITY TO DECOMPOSE FAT

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The view exists (Schreiber, 1902; Haag, 1928) that bacteria lose the ability to decompose fat when grown under laboratory conditions on ordinary nutrient media over a period of 18-21 months.

Seliber (1932) showed that some bacteria—Bacterium galleriae and Bacterium butyri I and II—which he isolated in 1915 retained the ability to decompose fat as late as 1931. At that time, he also established that two races of Pseudomonas aeruginosa (P. pyocyanea) continued to decompose fat for 6 years. Two nonsulfur purple bacteria isolated in 1920 behaved in the same manner. The latter two bacteria were studied with regard to the decomposition of vegetable oil by Pigulevskii and Kharik (1928). The same authors and Katsnel'son (1929) investigated B. galleriae and B. butyri I and II.

TABLE 1

Decomposition of Sunflower Oil (July, 1959)

| Cultures | Amount of oil, g | | | Oil consumed, % | Acid number | Saponification number | Iodine number |
|---|---|---|---|---|---|---|---|
| | initial | after expt. | consumed | | | | |
| 37-1 | 4.05 | 3.7470 | 0.3130 | 7.72 | 2.32 | 191.4 | 115.32 |
| B. butyri I | 3.912 | 3.6230 | 0.2890 | 7.39 | 3.10 | 190.2 | 118.75 |
| B. butyri II | 3.912 | 3.8980 | 0.0140 | 0.36 | 3.28 | 190.8 | 119.60 |
| B. galleriae | 3.966 | 3.9565 | 0.0095 | 0.24 | 30.06 | 193.2 | 81.84 |
| Control | 3.863 | 3.860 | 0.0030 | — | 2.21 | 191.03 | 120.02 |

TABLE 2

Decomposition of Olive Oil (December, 1959)

| Cultures | Amount of oil, g | | | Oil consumed, % | Acid number | Saponification number |
|---|---|---|---|---|---|---|
| | initial | after expt. | consumed | | | |
| 37-1 | 3.5264 | 3.2490 | 0.2774 | 7.86 | 3.4 | 196.92 |
| B. butyri I | 3 6488 | 3.4027 | 0.2461 | 6.74 | 6.9 | 197.20 |
| B. butyri II | 3.3932 | 3.3910 | 0.0022 | 0.065 | 0.97 | 196.84 |
| B. galleriae | 3.2722 | 2.980 | 0.2922 | 8.93 | 62.2 | 198.64 |
| Control | 3.0838 | 3.0836 | — | — | 0.86 | 196.33 |

The purpose of the present work is to demonstrate the retention of the ability of some bacteria to decompose fat after prolonged storage under laboratory conditions. Four bacteria were investigated: B. galleriae, B. butyri I and II, and bacterium No. 37-I, isolated from Leningrad soil in 1951.

The experiments were set up in Erlenmeyer flasks in liquid medium of the following composition (in %): $K_2HPO_4$-0.1, $MgSO_4$-0.3, $CaCl_2$-0.01, $(NH_4)_2HPO_4$-0.2, yeast water-5, sterile fat-2, pH 7-7.2. The medium was poured out in a thin layer, and sterile fat was added to the flasks after sterilization.

The duration of the experiment was three weeks. Olive and sunflower oils were used in the experiments.

The data of the experiments are given in Tables 1 and 2.

As shown by Tables 1 and 2, not all of the bacteria which we investigated retained the ability to decompose fat. The fat-splitting ability was retained in three species: B. butyri I, B. galleriae, and bacterium 37-I. B. galleriae, as before, split fat with a sharp rise in acid number.

In one experiment with olive oil and a culture of B. galleriae which we do not give, the acid number rose to 110.

Observations were also made in the laboratory on the growth of a number of bacteria in tubes of slanted mineral agar, to the bottom of which 2-3 ml of vegetable oil was added.

Inoculations were made either with a needle or a loop; bacterial growth with the formation of a film indicated their ability to consume the oil, or the loss of this ability.

At first, this method was used for the determination of acidity during the decomposition of vegetable oil. Using this method, Yakovleva tested for the ability to decompose vegetable oil in cultures No. 37, 45, 66, 172 and 174, which were subcultured on meat-peptone agar for three years, and obtained positive results. It should be pointed out that, for determining acidity, melted agar was used (not excessively hot) which contained the culture which had grown on it and residual vegetable oil.

Dobrovol'skaya observed the growth of 21 cultures (isolated from various soils) on the same medium for the purpose of establishing their ability to consume vegetable oil; it was found that 16 cultures retained this ability, while five lost it.

We should like to mention that all chemical analyses were performed by Yu. A. Dranitsyna, to whom we express our thanks.

## SUMMARY

It has been established that many bacteria which decompose fat at the time of their isolation can retain this ability when subcultured over a long period of time.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

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# VARIABILITY OF Bacillus mesentericus UNDER THE INFLUENCE OF THE PHYTONCIDES OF Chenopodium Botrys L.

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The variability of bacteria is a phenomenon which occurs widely in nature, and an extensive literature is devoted to this question. The investigations of certain authors concerning the effect of the phytoncides of garlic (Allium sativum L.) on some microbes have a more direct bearing on our work. Thus, Khetagurova (1947-1948) established that garlic juice diluted 1:50 causes morphological changes in the colonies of Pseudomonas phaseoli, Pseudomonas heteroceum, Pseudomonas campestris, and Pseudomonas tabacum and the disappearance of their pathogenic properties. The phytoncides of garlic also cause pronounced polymorphism in the tuberculosis bacterium (Nebolyubova, 1952), Corynebacterium diphtheriae (Yanovich, 1944), Clostridium perfringens (Bacillus perfringens) (Surnina, 1952), and Salmonella typhosa (Bacterium typhi abdominalis) (Sokolova and Voronina, 1952). Changes due to the effect of phytoncides have been described for staphylococci (Puzevskaya and Tikhonova, 1944), sarcinas, streptococci, and Proteus vulgaris (Bacterium proteus) (Karpov, 1944).

## EXPERIMENTAL DATA

While studying the antibacterial properties of the plant Chenopodium Botrys (fragrant goosefoot), we noticed that under the influence of its aqueous extract marked changes arose in the colony form of B. mesentericus. In connection with this, we decided to make a more thorough study of the morphological, cultural, and biochemical properties of B. mesentericus variants arising under the influence of Chenopodium Botrys.

We prepared an aqueous extract of the above-ground portion of the plant according to the method described in the Eighth Governmental Pharmacopoeia of the USSR. B. mesentericus was kept in a 75% aqueous extract of the plant for 24 hr in an incubator at 37 C, after which we studied the cultural, morphological, and biochemical changes in the microorganism. In order to study colony form, the B. mesentericus culture was plated on ordinary agar in Petri dishes after it was treated with the aqueous extract. It was found that the colonies that grew after treatment with phytoncide differed considerably in outward appearance from the colonies of the original strain. The colonies of the original strain were large, were whitish in color, dry, wrinkled, had uneven borders, and were stringy; the colonies of the variants obtained were very small, round, semitransparent, juicy, slimy, smooth, and had a fairly soft consistency (Fig. 1). When inoculated into meat-peptone broth, the original strain of B. mesentericus formed a whitish wrinkled pellicle on the surface of the broth, while the variants obtained produced even turbidity of the medium without the formation of a pellicle.

Cell morphology. The cells of B. mesentericus variants are considerably shorter and thicker than the original and stain less intensely with Mueller's stain (Figs. 2 and 3).

Biochemical activity. The variants of B. mesentericus are characterized by reduced biochemical activity: they peptonize milk and liquefy gelatin and coagulated blood serum more slowly than the original strain; they

• The greater part of our experiments were conducted at the Institute of Microbiology of the VMI in Sofia, in connection with which we offer our sincere thanks to Docent Sv. Vyrdarov.

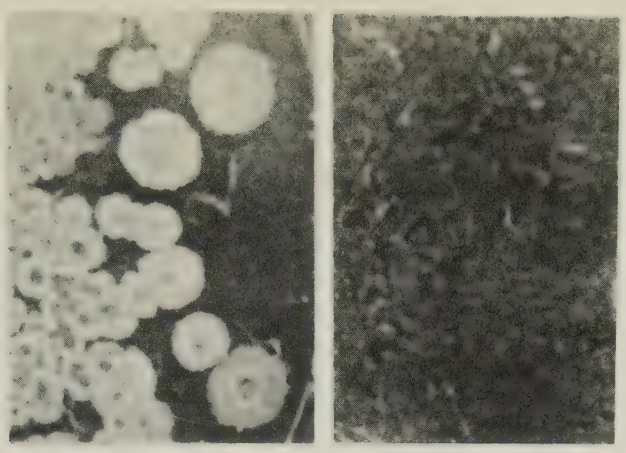

Fig. 1. Petri dishes inoculated with B. mesentericus. Left) original (control) strain; right) after 24 hr in 75% aqueous extract of Chenopodium Botrys (20 hr growth at 37 C).

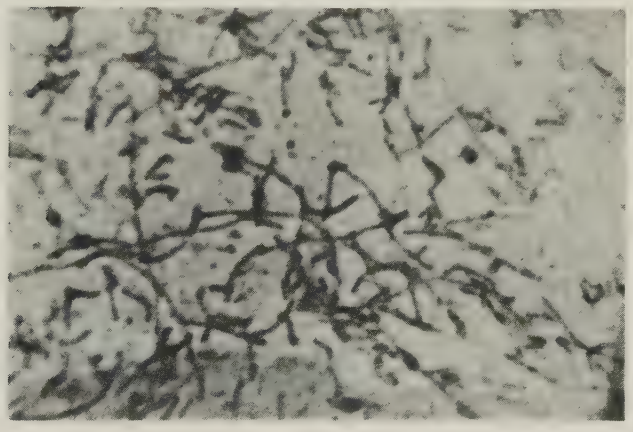

Fig. 2. B. mesentericus – original (control) strain (stained according to Mueller. Objective 90×; ocular 10×).

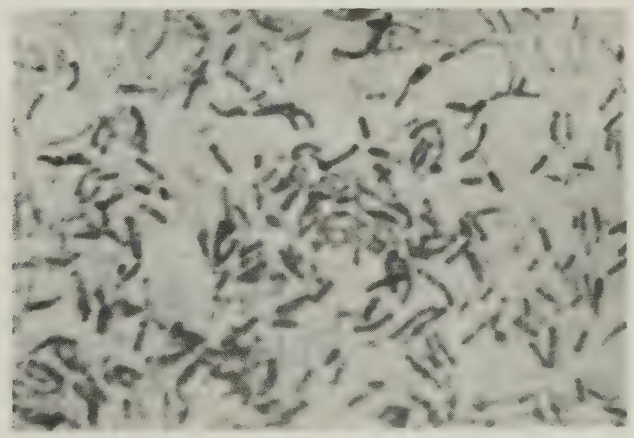

Fig. 3. B. mesentericus following treatment with 75% aqueous extract of Chenopodium Botrys (stained according to Mueller. Objective 90×; ocular 10×).

Cultural Characteristics of the Original and Newly Obtained Strains

| Substrate | Original strain | Variant obtained under the influence of phytoncides |
|---|---|---|
| Agar | Colonies large, whittish, dry, folded with uneven borders, stringy | Colonies small, round, semitransparent, smooth, fairly soft in consistency, slimy. Suggest the growth of nonsporogenous bacteria. |
| Broth | Medium transparent, whitish folded pellicle appears on the surface | Uniform turbidity, no pellicle formed |
| Gelatin | Stab inoculation: rapid, funnel-shaped liquefaction | Stab inoculation: slow, funnel-shaped liquefaction |
| Milk | Rapid peptonization | Slow peptonization |
| Coagulated blood serum | Rapidly liquefied | Slowly liquefied |
| Glucose | Rapid breakdown with acid production | Slow breakdown with acid production |
| Lactose | No decomposition | No decomposition |
| Mannitol | The same | The same |
| Sorbitol | Breaks down rapidly to the formation of acid | Breaks down slowly with acid production |
| Sucrose | Breaks down rapidly to the formation of acid | Does not split |

have a weaker enzymatic activity with respect to glucose and sorbitol, and lose their ability to break down sucrose (table).

## SUMMARY

Variants have been induced in Bacillus mesentericus by the phytoncides of Chenopodium Botrys which differ from the original culture in their cultural, morphological and biochemical characters.

The colonies are extremely minute, smooth and mucous. The cells of the variants are shorter and thicker and less reactive to Mueller staining. They cause uniform turbidity in the broth without formation of any film.

A peculiar property of the variant is its reduced biochemical activity: it peptonizes milk at a lower rate and liquefies gelatin and blood serum coagulum more slowly. Its enzymic activity with regard to glucose and sorbitol is slight and it does not decompose sucrose.

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# INDUCTION IN STAPHYLOCOCCI OF RESISTANCE TO STREPTOMYCIN, RIVANOL, AND COPPER BY CELL-FREE EXTRACTS

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The present work was concerned with the problem of obtaining cultures of microorganisms with previously selected properties.

We wished to demonstrate the genetic role of DNA. A number of articles by foreign investigators, beginning with Avery, MacLeod, and MacCarty (1944), have been devoted to this problem. The present work had as its aim the use of transformation as a method of rapidly obtaining forms of bacteria with the desired properties. The staphylococcus was chosen as the first model for this investigation because it is possible to transform the cells (Imshenetskii, Perova, Zaitseva, and Belozerskii, 1959). It was shown that with the aid of DNA isolated from staphylococci which were resistant to streptomycin, it was possible to induce in the original unadapted culture resistance to streptomycin. It turned out that in the original, unadapted culture of staphylococci resistant cells were encountered rarely. The number of mutations on an average was $1 \times 10^{-9}$. DNA in a concentration of 5 $\mu$g per ml raised the number of resistant cells to $4 \times 10^{-6}$, that is, increased it four thousand-fold. Data showed the specificity of action of DNA. We do not include any data on the induction in staphylococci or in any other bacteria of resistance to rivanol by the method of transformation. As for resistance to copper, a series of Japanese studies by Minagava, Yanagishima, Arakatsu, Nagasaki, and Ashida (1951, 1952, 1953) on induction of resistance to copper in Saccharomyces ellipsoideus, with the aid of cell-free extracts obtained from yeast adapted to copper which contained in the nutritive medium copper sulfate in the concentration of 1 $\mu$M per liter, are well known. The effect of the extract was expressed in the increase in the number of resistant cells from 77% in the original culture to 100% in the culture obtained by means of transformation. It was shown that RNA was responsible for the induction in the yeast of resistance to copper.

TABLE 1

Adapted Strains of Staphylococcus aureus

| Designation of the donor strain | Compound to which the strain was adapted | Concentration in MPA |
|---|---|---|
| RP | Rivanol | 0.04% |
| SP | Streptomycin | 100 thousand units/ml |
| RSP | Rivanol and streptomycin | 0.04%<br>100 thousand units/ml |
| MP | Copper | 0.005% |
| MSP | Copper and streptomycin | 0.005%<br>100 thousand units/ml |

The problem therefore is of considerable interest whether it is possible in a bacterium, in this instance in staphylococci, to induce resistance not only to a single substance, but to two chemical substances. Rivanol, streptomycin, and copper were utilized in this study for this purpose.

## EXPERIMENTAL DATA

Characteristics of the donor strain and the receipient strain. The work was carried out with a culture of Staphylococcus aureus strain 209. The original culture by successive transfer on meat-peptone agar containing gradually increasing concentrations of the given substances was adapted to rivanol, streptomycin, and copper and also to 2 compounds: rivanol and streptomycin, and copper and streptomycin.

TABLE 2

Number of Resistant Cells of the Original and Adapted Strains of Staphylococci on Different Media

| Medium | Concentration in MPA*** | Strains | Number of resistant cells (range) |
|---|---|---|---|
| MPA*** + streptomycin | 100,000 units/ml | Original SP; MSP; RSP | $n^{**} \cdot 10^{-11} - n \cdot 10^{-10}$ $5 \cdot 10^{-1} - 1.2$ |
| | 50,000 units/ml | The same | $n \cdot 10^{-10} - n \cdot 10^{-9}$ 1.0—1.2 |
| | 40,000 units/ml | » » | $n \cdot 10^{-9} - n \cdot 10^{-7}$ 1.0—1.2 |
| MPA + copper | 0.15%* | Original MP; MSP | $n \cdot 10^{-9} - n \cdot 10^{-7}$ $n \cdot 10^{-5} - n \cdot 10^{-3}$ |
| | 0.008% | The same | $n \cdot 10^{-6} - n \cdot 10^{-4}$ $n \cdot 10^{-3} - n \cdot 10^{-1}$ |
| | 0.005% | » » | $n \cdot 10^{-2} - n \cdot 10^{-1}$ 1 |
| MPA + rivanol | 0.05% | Original RP; RSP | $n \cdot 10^{-10} - n \cdot 10^{-9}$ $n \cdot 10^{-6}$ |
| | 0.04% | The same | $n \cdot 10^{-8} - n \cdot 10^{-6}$ 5 — 10 |

Note: *Concentration in % of copper sulfate; ** n — number of units; ***MPA — meat-peptone agar.

In Table 1 are given the conditions of the donor strain and the concentrations of the theraputic agent and of copper in the meat-peptone agar on which the adapted staphylococci gave 100% resistant cells.

The original culture of staphylococcus was used basically as the recipient strain. In Table 2 its characteristics are given according to the number of resistant cells at given concentrations of streptomycin, rivanol, and copper on meat-peptone agar. The number of resistant cells was determined as a ratio of the number of cells surviving a determined concentration of a chemical substance on hard nutritive medium to the total number of cells determined by their growth on meat-peptone agar. As can be seen from the given data, the number of resistant cells from the original culture grown on meat-peptone agar with streptomycin in the concentration of 100,000 units per ml was not significant and consisted in all of 1 to 10 resistant variants per 100 billion sensitive cells to streptomycin (from $\underline{n} \times 10^{-11}$ to $\underline{n} \times 10^{-10}$). The number of resistant cells increased when the concentration of streptomycin was decreased to 40,000 units per ml and remained quite low (1-100) per billion cells (from $\underline{n} \times 10^{-9}$ to $\underline{n} \times 10^{-7}$), while the adapted strains SP, MSP, and RSP on the very same medium gave 100 to 120% resistance. During the adaptation of the bacteria to copper it was difficult to evaluate the number of resistant cells which the original and the adapted strains gave. At copper concentrations of 0.015%, when the original strain gave 1 to 100 resistant mutants per billion cells (from $\underline{n} \times 10^{-9}$ to $\underline{n} \times 10^{-7}$), the donor strains MP and MSP did not give 100% growth. However, such growth did occur at a copper concentration of 0.005% and they grew in quantities of 1-100 per 100,000 cells (from $\underline{n} \times 10^{-5}$ to $\underline{n} \times 10^{-3}$). However, the requisite copper concentrations permitted a more clear-cut induction of resistance; in this instance, we obtained a maximal differentiation of 10,000 cells between resistance of the donor strain and the recipient strain. The transformed strain usually gave intermediate numbers of resistant cells between the recipient strain and the donor strain, approaching the latter under optimal conditions.

Method for obtaining cell-free extracts. To obtain cell-free extracts, donor strains were taken which were resistant to streptomycin, rivanol, and copper.

The cells were grown in flasks with 50 to 100 ml of meat-peptone broth at 37 C for 18 hr; the number of cells was determined by a Goryaev counter. Then 15 liters of meat-peptone broth were seeded with a culture of 50 million cells per ml and maintained at 37 C for 18 hr, after which the cells were separated by centrifugation

TABLE 3

Effect of Saline on the Resistance of Staphylococci to Streptomycin, Rivanol, and Copper

| Strain | Number of resistant cells per $10^8$ sensitive cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MPA + streptomycin 50,000 units/ml | | MPA + rivanol 0.4% | | MPA + copper 0.015% | | MPA + copper 0.008% | |
| | no. | % | no. | % | no. | | no. | % |
| Original | 40 | 100 | 5 | 100 | 8 | 100 | 100 | 100 |
| Original + saline | 64 | 160 | 6.4 | 128 | 13 | 162 | 300 | 300 |
| SP | | | 150 | 100 | 25 | 100 | 120 | 100 |
| SP + saline | | | 200 | 133 | 85 | 340 | 400 | 330 |
| MP | 20 | 100 | 2.5 | 100 | 1000 | 100 | 10000 | 100 |
| MP + saline | 30 | 150 | 3.2 | 129 | 3400 | 340 | 33000 | 330 |

at 3000 rpm for 25-30 min. The pellicle of cells obtained was washed free of meat-peptone broth with sterile distilled water. The whole operation was carried out under sterile conditions. The pellicle was suspended in 70% alcohol to precipitate the material and was kept at 4 C for 24 hr. The alcohol was separated by centrifugation and the precipitate was ground for 30 min with sterile glass powder which was added to the precipitate in equal quantity.

The destroyed cells were transferred to vials and placed into 15 ml of saline. The extracts were maintained at 4 C for 48 hr, after which the extract was twice centrifuged at 15-18,000 rpm for 10 min. The extract obtained was sterilized.

Method of carrying out experiments on induction of resistance in staphylococci to rivanol, streptomycin, and copper. The experiment on the induction of resistance in staphylococci to rivanol, streptomycin, and copper was carried out in the following manner: to 1 ml of culture fluid of the recipient strain was added an equal amount of the cell-free extract. It was ascertained that the greatest number of resistant cells were formed when the extract was added to cells at the end of the logarithmic phase of growth. This approximately corresponded to 5-6 billion cells per ml from the originally infected meat-peptone culture which contained 50 million staphylococci per ml. In this case the number of cells reached its maximum in 5 hr if the broth was infected with staphylococcal cells which were grown on meat-peptone broth, and after 10 hr the dose was transferred to cells on meat-peptone broth. As a control, saline was added instead of the extract. To determine the number of resistant cells the culture was carried for 24-72 hr on hard medium with streptomycin, rivanol and copper, and on meat-peptone broth.

Meat-peptone broth with added streptomycin, rivanol, or copper was prepared in the following manner: sterile streptomycin sulphate was added to melted and then cooled meat-peptone broth, after which the medium was poured into Petri dishes. An aqueous solution of rivanol was filtered through a membrane filter no. 1, and the desired amount was added to the meat-peptone broth. Copper sulfate was added to the medium before sterilization. Meat-peptone broth used for the preparation of the medium with rivanol and copper should be heated in the most careful manner. Each experiment was done in three replicates.

Experiments on transformation. We can judge from the increase in the number of resistant cells in the recipient strain after transformation in comparison with the number of mutations which were formed under the same conditions by the recipient strain to which was added saline (control), in order to assess the induction of resistance in staphylococci to the therapeutic agents and to copper by means of a cell-free extract. Saline, as is seen in Table 3, increased the number of resistant cells in comparison to streptomycin and rivanol in a ratio of 1.3 to 1.6, whereas copper did so in a ratio of 1.6 to 3.4.

It should be pointed out that many salts, among which is sodium chloride, possess defensive properties which inactivate the effect of streptomycin, rivanol, and copper on the cells. From these data it is also evident that the strain adapted to streptomycin gave approximately a thirty-fold greater number of resistant cells to rivanol in comparison with the original unadapted strain and also displayed a slight advantage in growth on media with

TABLE 4

Effect of Dilution of the Cell-Free Extract on the Decrease in the Number of Resistant Cells

| Variant of the experiment | Dilution of the extract | Number of resistant cells to streptomycin | Multiple of the increase of resistance in comparison with the original strain |
|---|---|---|---|
| Original strain | – | $2.3 \cdot 10^{-9}$ | 1 |
| Original strain plus saline | – | $3.0 \cdot 10^{-9}$ | 1.3 |
| Original strain plus extract SP* | 1/100 | $9.3 \cdot 10^{-9}$ | 4.0 |
| | 1/10 | $6.0 \cdot 10^{-4}$ | 260,000 |
| | 1 | $4.0 \cdot 10^{-2}$ | 15,200,000 |

*Extract SP obtained from staphylococci cells which were resistant to streptomycin.

TABLE 5

Induction in Staphylococci of Resistance to Streptomycin by means of Cell-Free Extract

| Variant of the experiment | Number of resistant cells (MPA plus streptomycin 50,000 units per ml) | Multiple of increase in the number of resistant cells in comparison with the control |
|---|---|---|
| Original plus saline (control) | $2.5 \cdot 10^{-10}$ | 1 |
| Original plus extract SP | $4.1 \cdot 10^{-2}$ | 164,000,000 |
| Original plus saline (control) | $3.0 \cdot 10^{-9}$ | 1 |
| Original plus extract SP | $3.1 \cdot 10^{-2}$ | 10,000,000 |
| Original plus saline (control) | $3.3 \cdot 10^{-9}$ | |
| Original plus extract SP | $6.6 \cdot 10^{-4}$ | 200,000 |

TABLE 6

Induction in Staphylococci of Resistance to Rivanol by means of Cell-Free Extract

| Variant of the experiment | Number of resistant cells (MPA plus rivanol 0.04%) | Multiple of increase in the number of resistant cells in comparison with the control |
|---|---|---|
| Original plus saline (control) | $2.2 \cdot 10^{-8}$ | 1 |
| Original plus extract RP* | $9.2 \cdot 10^{-5}$ | 4180 |
| Original plus saline (control) | $3.2 \cdot 10^{-8}$ | 1 |
| Original plus extract RP | $6.4 \cdot 10^{-5}$ | 2000 |
| Original plus saline (control) | $6.4 \cdot 10^{-8}$ | 1 |
| Original plus extract RP | $2.4 \cdot 10^{-5}$ | 375 |

*Extract RP obtained from staphylococci cells which were resistant to rivanol.

copper. However, the strain adapted to copper gave on media with streptomycin and rivanol no increase, but a decrease, in the number of resistant cells by twofold.

In diluting the extract ten- and a hundredfold, the number of resistant cells was sharply decreased. Dilution of the extract a hundredfold almost approached the effect of saline (see Table 4).

Induction in Staphylococci of Resistance to Copper by means of Cell-Free Extract

| Variant of the experiment | Number of resistant cells (MPA plus copper 0.015%) | Multiple of increase in the number of resistant cells in comparison with the control |
|---|---|---|
| Original plus saline (control) | $1.2 \cdot 10^{-7}$ | 1 |
| MP plus saline | $2.4 \cdot 10^{-3}$ | 20000 |
| Original plus MP extract* | $1.2 \cdot 10^{-3}$ | 10000 |
| Original plus saline (control) | $8.0 \cdot 10^{-8}$ | 1 |
| MP plus saline | $2.0 \cdot 10^{-4}$ | 2500 |
| Original plus MP extract | $2.0 \cdot 10^{-5}$ | 250 |
| Original plus saline (control) | $1.3 \cdot 10^{-9}$ | 1 |
| MP plus saline | $1.0 \cdot 10^{-5}$ | 7538 |
| Original plus MP extract | $1.2 \cdot 10^{-6}$ | 923 |

*Extract MP obtained from staphylococci cells which were resistant to copper.

By means of a cellular extract obtained from strains adapted to streptomycin we suceeded in bringing about transformation which was expressed by the increase in the number of resistant cells in comparison with the control (original strain plus saline). From the data given in Table 5 it can be seen that the number of resistant cells in comparison with the control increased tens, and even hundreds, of millions of times. In the first experiment the number of resistant cells approached 4% of the total number of cells subjected to the effect of the extract. We did not succeed in obtaining such a significant increase in the number of resistant cells during the induction of resistance to rivanol. The number of resistant cells after the addition of the extract increased only hundreds and thousands of times (see Table 6).

The effect of the extract obtained from staphylococci cells which were resistant to copper can enhance the resistance of the recipient strain 10,000-fold. As was already noted above, the determination of the number of resistant cells was carried out on a medium with a high content of copper in meat-peptone agar, which permitted a more clear-cut establishment of the induction of resistance to copper in these cells. Tables 7 and 9 show the number of resistant cells which were obtained on meat-peptone agar, with copper in a concentration of 0.015% in strains adapted and not adapted to copper. With the cell-free extract, the number of resistant cells in the recipient strain approached the number of cells which were found in the donor strain on the same medium, and in separate instances (Table 9, Experiment 3) even exceeded this number. Such cases were not rare, and this fact undoubtedly represents something which requires further study. An attempt was made to induce resistance to two compounds in the recipient strain (in this instance to streptomycin and rivanol and to streptomycin and copper) by means of the extracts obtained from cultures of organisms adapted to two compounds. The data on these experiments are given in Tables 8 and 9. The determination of the number of resistant cells showed that it is possible to induce in the culture resistance to rivanol and streptomycin and to streptomycin and copper at one and the same time.

Either the effect of the cell-free extract is such that the single transformed cell becomes resistant to the two compounds, or the culture represents in itself a mixture of cells, one of which is resistant to streptomycin and another to rivanol or copper. These questions are in need of further and more profound investigation. Also an even more interesting theoretical problem requires a solution: that is, is DNA responsible for the induction of resistance to rivanol and to copper, or is the transformation of resistance to copper realized by RNA, as was shown by Minagava (1955) in regard to the molds.

We wish to express our deep appreciation to Professor A. A. Imshenetsky for his guidance in this work.

TABLE 8

Induction in Staphylococci of Resistance to Streptomycin and Rivanol by means of Cell-Free Extract

| Variant of the experiment | MPA plus streptomycin 50,000 units per ml | | MPA plus rivanol 0.04% | |
|---|---|---|---|---|
| | Number of resistant cells | Multiple of the increase in resistance in comparison with the control | Number of resistant cells | Multiple of the increase in resistance in comparison with the control |
| Original plus saline (control) | $6.0 \cdot 10^{-9}$ | 1 | $5.0 \cdot 10^{-8}$ | 1 |
| Original plus extract RSP* | $3.9 \cdot 10^{-5}$ | 6500 | $4.6 \cdot 10^{-5}$ | 912 |
| Original plus saline (control) | $1.6 \cdot 10^{-9}$ | 1 | $3.1 \cdot 10^{-8}$ | 1 |
| Original plus extract RSP | $6.6 \cdot 10^{-6}$ | 4150 | $8.0 \cdot 10^{-5}$ | 2510 |
| Original plus saline (control) | $2.0 \cdot 10^{-9}$ | 1 | $3.0 \cdot 10^{-7}$ | 1 |
| Original plus extract RSP | $8.0 \cdot 10^{-7}$ | 400 | $9.1 \cdot 10^{-5}$ | 303 |

*Extract RSP obtained from staphylococci cells which were resistant to rivanol and streptomycin.

TABLE 9

Induction in Staphylococci of Resistance to Streptomycin and Copper by means of Cell-Free Extract

| Variant of the experiment | MPA plus streptomycin 50,000 units per ml | | MPA plus copper 0.015% | |
|---|---|---|---|---|
| | Number of resistant cells | Multiple of increase in resistance in comparison with the control | Number of resistant cells | Multiple of increase in resistance in comparison with the control |
| Original plus saline (control) | $3.0 \cdot 10^{-8}$ | 1 | $1.2 \cdot 10^{-7}$ | 1 |
| MSP plus saline | | | $2.2 \cdot 10^{-4}$ | 1830 |
| Original plus extract MSP* | $1.2 \cdot 10^{-4}$ | 4000 | $2.0 \cdot 10^{-4}$ | 1660 |
| Original plus saline (control) | $5.3 \cdot 10^{-9}$ | 1 | $9.0 \cdot 10^{-8}$ | 1 |
| MSP plus saline | | | $2.6 \cdot 10^{-3}$ | 28800 |
| Original plus extract MSP | $2.0 \cdot 10^{-3}$ | 38000 | $8.7 \cdot 10^{-4}$ | 9660 |
| Original plus saline (control) | $1.1 \cdot 10^{-8}$ | 1 | $1.0 \cdot 10^{-7}$ | 1 |
| MSP plus saline | | | $2.0 \cdot 10^{-4}$ | 2000 |
| Original plus extract MSP | $1.0 \cdot 10^{-2}$ | 1000000 | $4.2 \cdot 10^{-2}$ | 420000 |

*Extract MSP obtained from staphylococci cells which were resistant to copper and streptomycin.

## SUMMARY

1. By means of cell-free extracts obtained from staphylococci cells adapted to streptomycin, rivanol and copper, it is possible to induce resistivity to these compounds in the cells of the original nonadapted staphylococcus culture.

2. After treatment with the extract, the number of resistant cells increased, in comparison with the control, by tens and hundreds of millions of times for streptomycin, by hundreds and thousands of times for rivanol, and by thousands and tens of thousands of times for copper.

3. Extracts obtained from staphylococci adapted to two chemical compounds, viz., streptomycin and rivanol or streptomycin and copper, induce in the nonadapted strain resistance to both compounds simultaneously.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

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# HETEROGENEITY OF THE CELL WALLS OF Actinomyces violaceus AERIAL MYCELIUM

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The application of bright field and polarized bright field light techniques to the study of actinomycete aerial mycelium structure [Krasilnikov and Kalakutskii, 1959] has enabled us to postulate heterogeneity in the cell wall structure of the aerial mycelium in these organisms. The present article is devoted to a more detailed investigation of this question.

## METHOD

Hyphae of aerial mycelium from a 4-5 day culture of Actinomyces violaceus strain 829 kindly supplied by O. I. Artamonova were the objects of our investigation. The same cells were observed in different lights, staining and restaining.

All observations were made with an MBI-6 microscope. A "Kiev" camera was used for photography. Black and white photographs were taken on mikrat-200 film, color photographs on Agfacolor film. Bright field examination was performed with a 40 × epiobjective.

Preparation of imprint-preparations. Cultures of A. violaceus were inoculated onto cellophane leaflets placed on the surface of potato agar. After 4-5 days of incubation at 27 C the cellophane leaflets bearing cultures were removed from the surface of the nutrient medium and were placed for a day in a desiccator over a saturated solution of $NH_4Cl$ at room temperature (at such temperature the atmosphere over such a saturated solution contains 79.3% water vapor). Then imprint preparations were made on fat-free glass objectives. In this manner we succeeded in obtaining good reproductions of the material which would subsequently be placed under the microscope. In 4-5-day cultures the growth of A. violaceus on potato agar gave sufficiently abundant aerial mycelia which were only entering the spore-forming stage. In preliminary experiments we established that unfixed and heat-fixed cells behave identically in relation to the properties which interested us. Therefore, in subsequent work, preparations which were carefully heat-fixed were used.

Staining. Where it was indicated, the mycelium was stained with Sudan B (black). The stain was prepared in the following manner: a saturated (at room temperature) alcoholic solution of Sudan B was chilled in the refrigerator to +4 deg, then filtered through folded filter paper (white ribbon). To the filtered solution was added an equal quantity of n-butanol. We stained with the resulting solution for 5 hr at room temperature. We examined the preparation in water.

If the cells had to be washed clean of Sudan B for subsequent staining by the Pekarskii method, the preparations were placed overnight in n-butanol at room temperature. Microscopic observations showed that the stain is completely washed off in these circumstances.

For Pekarskii staining the preparations were hydrolyzed in 1 N HCl at 60 C for 8 min and, after rinsing in water, were stained with Giemsa at 37 C for 1 hour (1 drop commercial stain in 1 ml distilled water adjusted to pH 7.0 with neutral red). The preparations were examined in water.

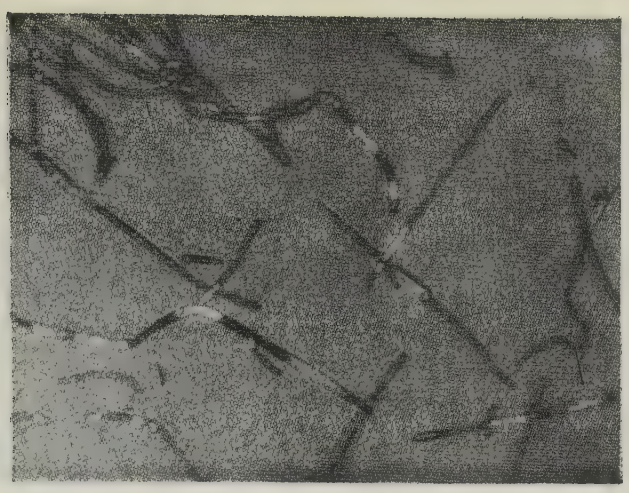

Fig. 1. Group of hyphae of A. violaceus aerial mycelium. Imprint-preparation on glass. Heat fixation. Bright field light. 1800 ×.*

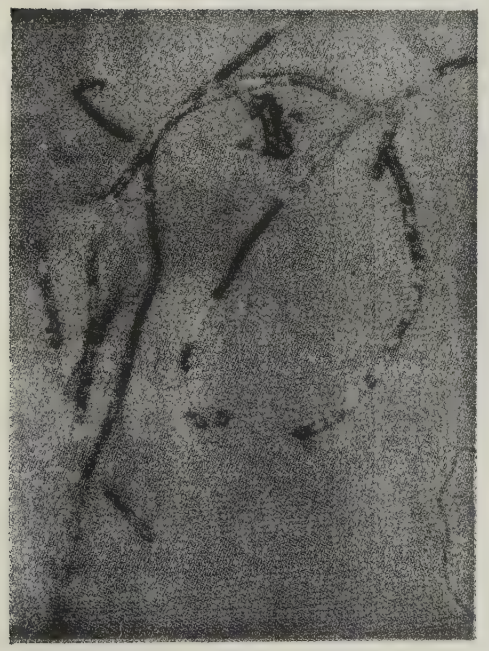

Fig. 2. Same, another group of cells. 1800×.*

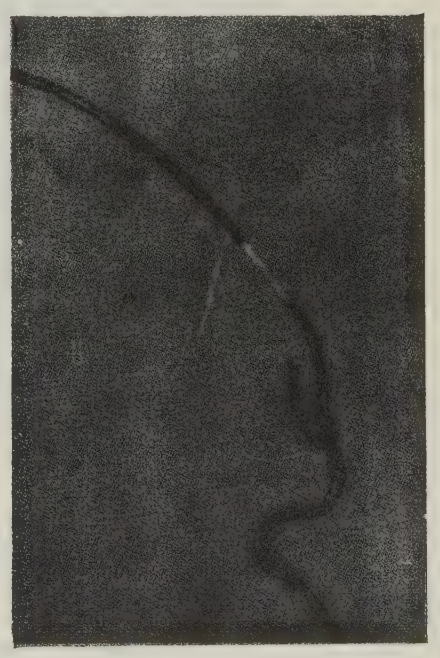

Fig. 3. Same, isolated hypha. 1800 ×.*

## RESULTS AND DISCUSSION

Figure 1 presents a typical imprint-preparation of A. violaceus aerial mycelium examined by bright field light. The heterogeneous staining of the mycelium is striking—some sections show the most varied colors (gold, orange, red, blue-green), while other parts are mostly unstained. It has been proposed [1] that such colored "fluorescence" of sections of the aerial mycelium is related to interference phenomena and reflections from the surface of sections of the medium with different refractive indices. We suggest that this surface is heterogeneous in certain properties.

It seems of interest to elucidate the specific property by which this heterogeneity is manifested and to see if there is a correlation between the different properties characteristic of the cell wall. In the present paper one group of cells was observed: 1) in reflected light (color print obtained); 2) in reflected polarized light with crossed

*Figures 1, 2, and 3 are reproductions of color originals. Hence, some detail has been lost – Publisher.

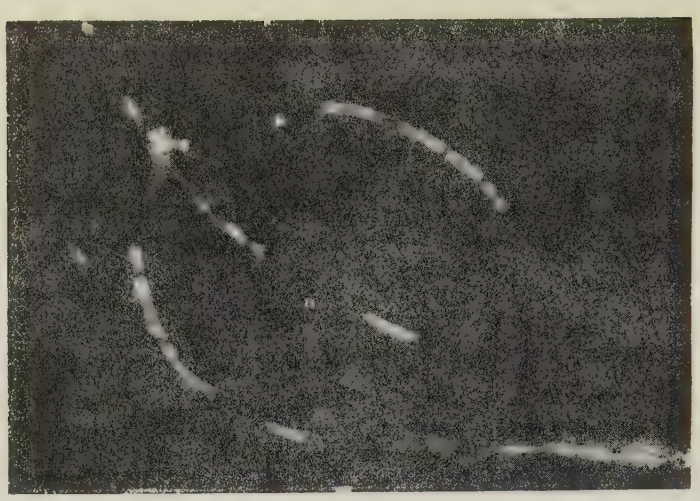

Fig. 4. Same cells as in Fig. 2. Reflected polarized light (crossed polaroids).

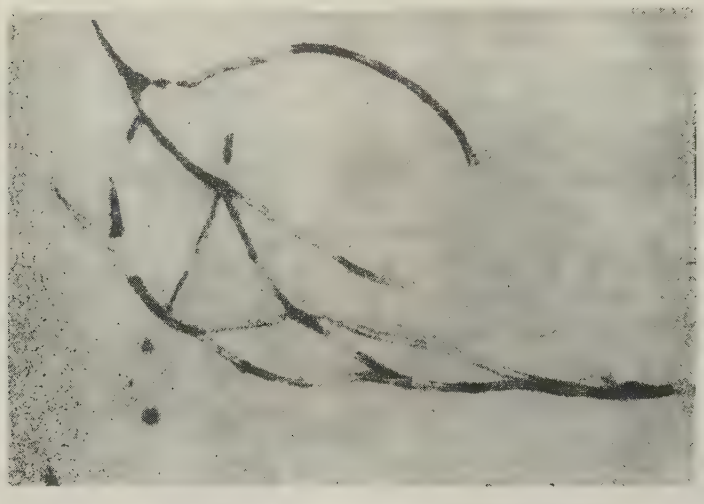

Fig. 5. Same cells as in Fig. 2. Stained with Sudan B. Transmitted light.

polaroids; 3) in transmitted light after Sudan B staining; 4) in reflected and reflected polarized light after washing out the Sudan B with butanol; 5) in transmitted light after staining by the Pekarskii technique.

The results are presented in Figs. 2-11. Compare imprints in Figs. 2, 4, 5 and 6. The following pattern is distinctly recognized: the sections of mycelial filaments which are tinted different colors on examination in reflected light correspond to the parts of the mycelial wall which display optical anisotropy reflected polarized light, crossed polaroids). Sections which stain with Sudan B correspond exactly to these parts. This last point confirms the suggestion expressed earlier [Krasil'nikov and Kalakutskii (1959); Erikson (1947) that we are here meeting substances of lipoid nature. Comparing the pictures in Figs. 2, 4, and 5 with Fig. 6 we can see that any structure inside the cells which has the property of staining with nuclear stain does not correspond to the sections of the mycelial wall which have special characteristics in reflected and reflected polarized light and in staining with Sudan B. In this regard a negative result is also obtained in experiments which involve cultivation of actinomycetes in a medium containing 0.01% triphenyltetrazol and subsequent observation of the localization of triphenyltetrazol in the mycelium. These correlations appear more clearly in Figs. 3, 7, 8, and 9, where we chose to illustrate long hyphae of the aerial mycelium in which only separate sections of the cell wall are notable for the properties enumerated.

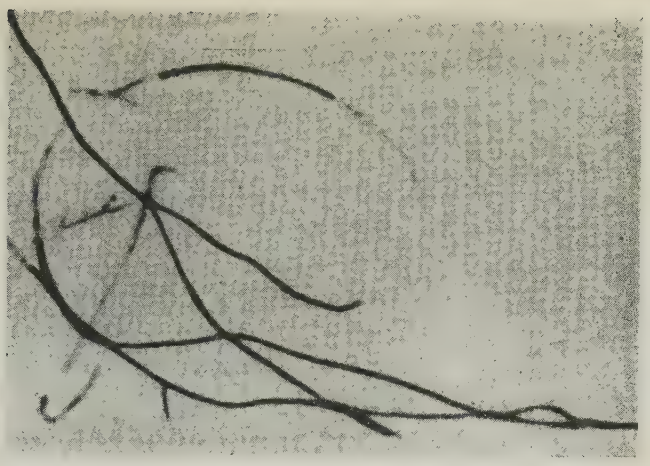

Fig. 6. Same cells as in Fig. 2. Pekarskii stain. Transmitted light.

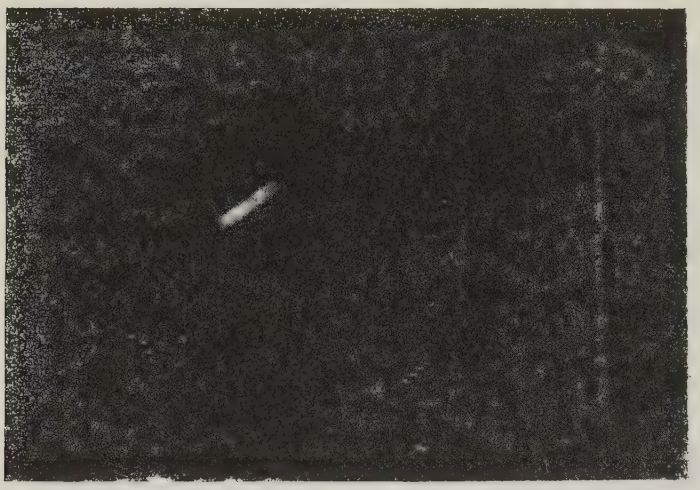

Fig. 7. The same cell as in Fig. 3. Reflected polarized light (crossed polaroids).

The ability to give colored images in reflected light is renewed in cells stained with Sudan B after washing out the stain with butanol. The ability to manifest optical anisotropy in reflected polarized light after such a procedure is greatly diminished.

Not only the cell walls of aerial mycelia, but also cell walls of various spores, even those apparently formed in a single chain, have heterogeneous features. This heterogeneity is also mainfested in the capacity to form colored images of various hues, or not to form colored images in reflected light, as well as in the property of optical anisotropy and stainability with Sudan B. In Figs. 10 and 11 are presented pictures of the spores of A. violaceus 829.

We should note that the suggested heterogeneity of aerial mycelium cell walls is likely to be real in view of data [Lesheval'e and Tikhonenko (1960)] concerning the fact that the cell walls of a number of spores from actinomycete sporangia react differently in a vacuum under the electron microscope. On the other hand, electrophoretic studies of the surface layers of Mycobacterium phlei cell walls [Rideal and Adams (1958)] also permit us to suggest a heterogeneity of substances in the cell walls of this organism. Ponder [Ponder (1949)] has postulated the presence of micromosaic structures in the lipoprotein membrane of the erythrocyte.

In conclusion, we wish to express our gratitude to Prof. N. A. Krasil'nikov for his guidance in this work.

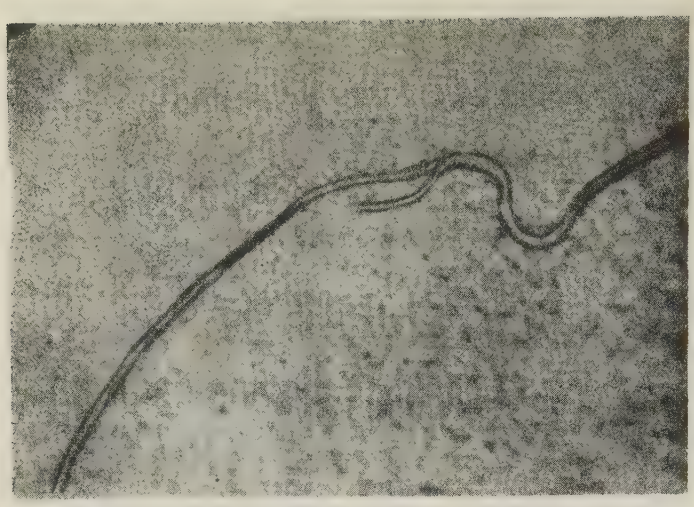

Fig. 8. The same cell as in Fig. 3. Sudan B stain. Transmitted light.

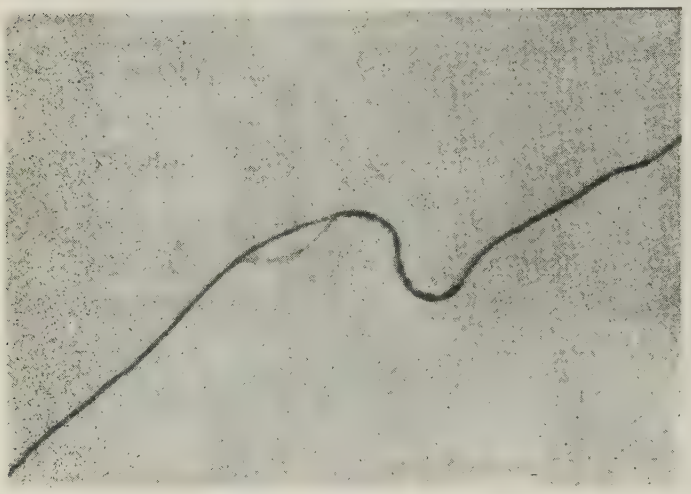

Fig. 9. The same cell as in Fig. 3. Pekarskii stain. Transmitted light.

Fig. 10.

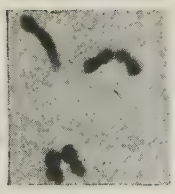

Fig. 11.

Fig. 10. Group of spores in reflected polarized light (crossed polaroids).

Fig. 11. The same group of spores. Sudan B stain. Transmitted light.


## SUMMARY

The cell wall of Actinomyces violaceus aerial mycelium is heterogeneous. Sections within it clearly manifest different properties in relation to the following features: 1) Ability to reveal or not to reveal coloration in reflected light; 2) ability to manifest optic anisotropy in polarized reflected light; 3) stainability with Sudan B. These features are interrelated. Sections of the cell walls which possess special properties with respect to these three features do not correspond to those parts of the mycelial contents which stain with nuclear dyes.


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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

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# MORPHOLOGY AND CYTOLOGY OF *Actinomyces streptomycini* AS A FUNCTION OF THE COMPOSITION OF THE MEDIUM

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Microbiological Institute, Academy of Sciences, USSR
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January-February, 1961
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In connection with the wide application of streptomycin the physiology of its producer, Actinomyces streptomycini, is under detailed investigation. Less attention has heretofore been paid to the study of the morphology and cytology of this culture.

The life cycle of A. streptomycini has been studied by Carvajal [1946, 1947], Klieneberger-Nobel [1947], and McGregor [1954].

In the Soviet literature an analysis of the cytological and biochemical changes in the mycelia during biosynthesis of streptomycin under deep culture conditions was performed by Prokof'eva-Bel'govskaya [1956, 1957]. In these studies it was shown that the growth of A. streptomycini takes place in two stages. Each phase is distinct not only in mycelial morphology, but also in the degree of protoplasmic basophilia, isoelectric point, RNA and DNA content, dimensions of nuclear elements and mycelial amino acid composition.

The following phases of growth and development of A. streptomycini under conditions of deep culture are distinguished: 1) Germination of inoculated material; 2) intensive mycelial growth with homogeneous basophilic cytoplasm; 3) slowing of growth, decrease in basophilia and differentiation of cytoplasm; 4) spore formation and disintegration of mycelia.

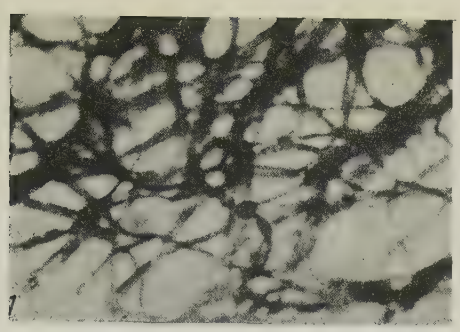
With respect to the mycelial content of RNA and DNA, we observe that during the growth of actinomycetes the intracellular RNA decreases and the DNA content increases.

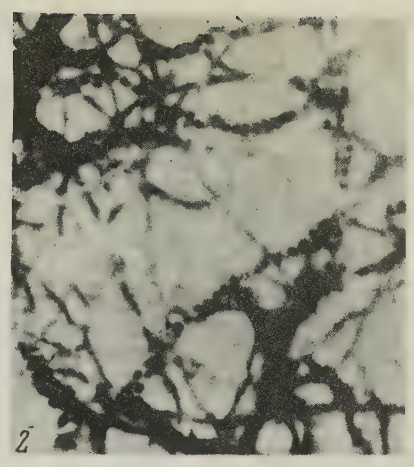
The present paper is devoted to a study of the morphology and cytology of A. streptomycini in media composed of amino acids, derived pyrrols and dicarboxylic acids.

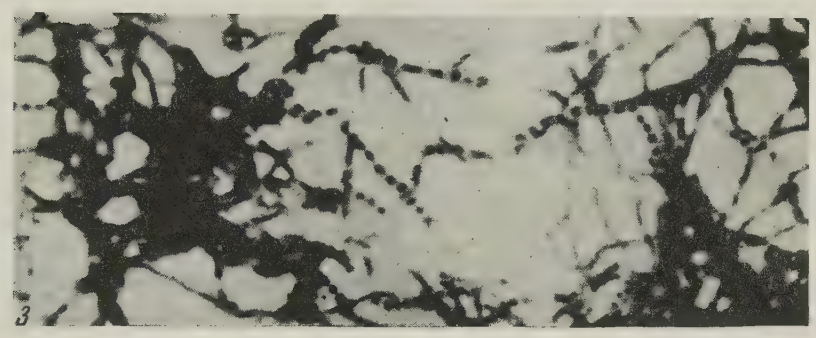
## METHOD

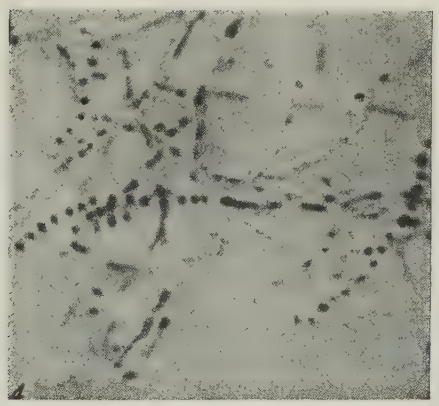
The experiments were performed on the LS-1 strain obtained from the Kiev selection station in a medium of the following composition (in %): Glucose-2; $(NH_4)_2SO_4$ - 0.3; NaCl - 0.25; $KH_2PO_4$ - 0.05; $CaCO_3$ - 0.3; distilled water. To this medium was added either 2% soybean meal or proline [Shaposhnikov and Kazanskaya, 1959], or histidine, lysine and succinamide or succinimide [Shaposhnikov and Kazanskaya, 1959], or histidine, lysine and dicarboxylic acids [Kazanskaya and Peltava, 1959]. The media were inoculated with 72-96 hr cultures of mycelia. The composition of the medium in inoculated and control flasks was always identical. The culture was maintained at 26-28 C on an agitator which described 240-250 rpm.

Microscopic study of the preparations was made at 600× magnification. The preparations were first dried, then fixed in Carnoy's fluid and stained with methylene blue (1: 1000) for microbiological control, estimation of the degree of basophilia and determination of the RNA content. For a qualitative assay of DNA the preparations were worked up and stained by the Feulgen method or Romanovsky-Giemsa, followed by counterstaining with gentian violet or light green [Peshkov, 1955].









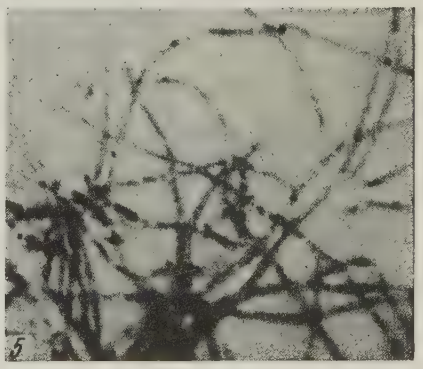


Fig. 1. Three-day culture in medium containing ammonium sulfate. Methylene blue. 600 ×.

Fig. 2. Three-day culture in medium containing histidine, lysine, malic and succinic acids. Methylene blue. 600 ×.

Fig. 3. Three-day culture in medium containing histidine, lysine, succinic and fumaric acids. Methylene blue. 600 ×.

Fig. 4. The same. Feulgen stain. 600 ×.

Fig. 5. Three-day culture in soybean meal medium. Methylene blue. 600 ×.

We compared the development of streptomycin production in different synthetic media to its development in soybean medium in which the change in growth conditions for A. streptomycini proceeds smoothly and consistently.

According to the nature of the life cycle, actinomycetes are divisible into groups which have one or another particular type of growth and development [Prokof'eva-Bel'govskaya and Zolotnitskii, 1955]. Under one set of conditions actinomycetes are distinguished by rapid growth and development, under other conditions rapid growth

but slow maturation or, on the other hand, slow growth but rapid maturation. Finally one encounters cases of slow growth and slow maturation.

The inferior growth in certain media, as a rule, is correlated with abnormally developed mycelia, poor differentiation of cytoplasm, an increased amount of metachromatin, great or, contrariwise, extremely faint, cytoplasmic basophilia all throughout the growth of the mycelia.

Certain of such differences in the rates of growth and maturation of strain LS-1 were observed in our experiments in a number of synthetic media.

In the first experiment of A. streptomycini cultivation in a medium which contained ammonium sulfate as the single nitrogen source, antibiotic activity was 102 μ g/ ml or 7% of the amount of streptomycin extracted from soybean meal medium. The growth of strain LS-1 was very slow. Up to the fifth day the mycelia consisted of small, solitary microcolonies (Fig. 1), which only on the sixth day of cultivation showed slight interlacing of peripheral hyphae to form rather friable mycelial networks. A high degree of cytoplasmic basophilia was characteristic of these mycelia and was accompanied by poor differentiation. For 3-6 days the plasm remained divided into very long, bacilliform segments. Only in seven-day culture did the cytoplasmic basophilia diminish and were sharply basophilic prospores formed at the tips of individual hyphae, as is characteristic for the beginning of stage 4 in the development of actinomycetes on soybean meal medium.

The cultivation of A. streptomycini in synthetic medium containing proline was characterized by comparatively slow growth and particularly by slow development. The total mycelial content for the LS-1 strain reached 730-820 mg % on the fifth to sixth day.

Three-day mycelia under the microscope showed medium-sized microcolonies. The cytoplasm of the even-contoured hyphae showed basophilia well but its differentiation was very poor (beginning stage 2): the hyphae were divided into long fragments. The mycelia entered the stage of advanced cytoplasmic differentiation and decreased basophilia only on the sixth day of development; at the same time, as in many other media, autolysis of the mycelium was occurring.

In the given instance the slow growth and development of the actinomycetes in proline medium was accompanied by the formation of 1345 μ g/ ml of antibiotic.

In media to which succinamide or succinimide had been added as the single organic nitrogen source, little nucleide was formed on the third to fourth days and during the following three days the microscopic picture did not change. With the addition of pyrrole group compounds to histidine and lysine, the formation of nucleides could be seen in preparations on the third and fourth days, in quantities which increased during the subsequent days of actinomycete development. Since the cell DNA content is related to nucleide formation, we may conclude that the quantity of DNA in the mycelia is increased on the addition of basic amino acids to the medium.

In a medium containing histidine and lysine as organic nitrogen sources, the differentiation of the mycelium was observed to begin only on the third day and nucleide formation took place from the fourth day. In this medium 853 μ g/ ml of streptomycin was formed by a total mycelial content of 654 mg%.

With A. streptomycini cultivation in a medium containing histidine, lysine and dicarboxylic acids another type of growth and development was observed from that which took place in media containing a number of pyrrol compounds and, in particular, proline.

The great rapidity of accumulation of the biomass with a subsequent cessation and the especially rapid development is characteristic of the LS-1 strain in synthetic media containing malic and succinic acids (Fig. 2) or succinic and fumaric acids (Fig. 3). This type of growth and development is often accompanied by a high yield of antibiotic [Prokof'eva-Bel'govskaya and Zolotnitskii, 1955].

The quantity of streptomycin in media containing malic and succinic acids comprised 113% of the antibiotic yield in soybean meal medium, or 1959 μ g/ml; in media containing succinic and fumaric acids 1468 μ g/ ml of antibiotic was formed. The total mycelial content in both media was identical: 633 mg%.

Already on the third day of cultivation the culture of A. streptomycini was in stage 4, accompanied by intensive spore formation, spores being disposed in continuous chains on long hyphae. In medium containing succinic and fumaric acids the actinomycete culture was in the stage of partial autolysis; sporulating fragments of

hyphae could be seen in the preparations. Prospores on the hyphae of such mycelia were very large.

The characteristic developmental feature of A. streptomycini in dicarboxylic acid-containing media is the high capacity for the synthesis of a large amount of DNA in the early stages of development. The hyphae of three-day mycelia contained numerous important nucleides with a distinct nuclear reaction (Fig. 4). With the Feulgen technique they were intensely stained a violet-purple color. The presence in the cytoplasm of a large amount of nuclear elements evidently stimulated the disintegration of young hyphae into numerous spore-containing fragments.

A strong cytoplasmic basophilia attested to the still considerable content of ribonucleic acid.

The nature of actinomycete development in media containing any single organic acid was distinguished by a delay in spore formation, whereas at the same time in nutrient media containing a mixture of these same acids we may observe the early onset of abundant spore formation.

The vegetative stage in the actinomycete life cycle in malic or fumaric acid-containing media is longer; the formation of prospores began toward the sixth to seventh day, while in succinic acid-containing medium the mycelia passed out of the vegetative state into the autolytic stage, omitting the stage of spore formation.

We find it interesting to note that the microscopic picture of the mycelia of the LS-1 strain during growth in media containing one of the organic acids was distinguished by the form of the hyphae in the mycelium: long and slightly curved hyphae became thickened, distorted "curls" with hook-like thickenings at the ends.

For comparison, Fig. 5 shows three-day cultures grown in soybean meal medium. These consist mainly of hyphae in which spores are only beginning to form.

Thus, the investigation we carried out on the cultivation of A. streptomycini in synthetic media containing different organic compounds has enabled us to make known certain morphological and cytological features of the LS-1 strain.

In conclusion, we express our appreciation to Prof. V. N. Shaposhnikov, under whose guidance this work was accomplished.

## SUMMARY

The LS-1 strain of Actinomyces streptomycini undergoes age changes during streptomycin biosynthesis in submerged culture which are manifested in changes in mycelial morphology, the physiological properties of the culture and the chemical composition of the cytoplasm, these changes depending on the composition of the medium and being related to the capacity for antibiotic formation.

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# ACTINOMYCETES OF THE ORANGE GROUP

## I. CULTURAL-MORPHOLOGICAL CHARACTERISTICS

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Actinomycetes which have orange-colored colonies, pink or orange aerial mycelium, but which do not impart their color to the medium form a distinct group of definite interest.

According to data in the literature [Krasil'nikov, 1949], the orange actinomycetes are rarely encountered in nature. These cultures were formerly distinguished as a single species, Actinomyces aurantiacus [Krasil'nikov, 1941]. At present, after careful study of a great number of cultures we have ascertained that those actinomycetes once regarded as A. aurantiacus represent a group of different species. The aim of this paper was to study and characterize the features of the different representatives in this group.

We studied 62 cultures of actinomycetes isolated from different sample soils in the Chinese People's Republic and the Soviet Union, cultures which are in the collection of the Krasil'nikov Laboratory of the Microbiological Institute, Academy of Sciences, USSR. The degree of their dissemination in the soils is indicated in Table 1.

From Table 1 it is apparent that orange actinomycetes inhabit soils of different types and different geographical zones. They are found in soils at Pekin, Kun'min region of the Chinese People's Republic and in the Pamirs, in soils from the Crimea, Caucasus and other regions of the USSR.

The greatest distribution is in the red soils, chernozem, serozem, and brown mountain bog soils of Kandara. They are rarely encountered in podzol or snow-covered soils. We did not find them in river, lake or other water silts.

We studied the structure, mycelial growth, and formation of fruiting organs of each strain as well as the cultural, physiological and antibiotic properties, specific antagonisms and other peculiarities.

We used the methods of the Krasil'nikov laboratory. Spore structure was studied under the electron microscope [Lesheval'e and Tikhonenko, 1960].

In the present article only data on the morphological-cultural features of orange actinomycetes are presented.

In studying the morphological-cultural features of orange group actinomycetes, first described by Krasil'nikov [1941, 1949] as A. aurantiacus, we found distinctions in the structure of spore-forming hyphae and in the cultural characteristics. On this basis we have divided the 62 orange actinomycetes into six subgroups. A description of these subgroups follows.

Subgroup I. In subgroup 1 are included 22 strains: Nos. 3024, 585, 612, 738, 2819, 2831, 2839, 2857, 2842, 2849, 2827, 2866, 2876, 2918, 3017, 3018, 3026, 3027, 3045, 3048, 3064, 2886.

In medium I [Krasil'nikov, 1950] containing glucose the growth was good. Colonies are uneven, bright-orange. Aerial mycelia are absent or very poorly developed, orange or pink. The medium is unstained. The

TABLE 1

Distribution of Actinomycetes of the Orange Group

| Subgroup | Strain No. | Habitat | Year isolated |
|---|---|---|---|
| 1 | 3024, 2819, 2831, 2839<br>2857, 2842, 2849, 2827<br>2866, 2876, 2886, 2918<br>3017, 3018, 3026, 3027<br>3045, 3048, 3064 | Red earth soil near Sishan (west mountain) Kun'min, CPR | 1957 |
| 2 | 585 | Medium loam, brown soil, USSR | 1959 |
| | 612 | Kandara, eroded bog soil, USSR | 1959 |
| | 738 | Kandara, brown bog soil, USSR | 1959 |
| | 5008, 5012, 5007, 5004 | Red earth soil, Kun'min, CPR | 1957 |
| | 205 | Kandara, brown bog soil, USSR | 1959 |
| | 2933 | Tashkan serozem, USSR | 1954 |
| | 1149 | Yalta, Crimea, soil from beneath a vineyard, slatey, USSR | 1950 |
| | 1510 | Timiryazevskaya podzol, Moscow, USSR | 1952 |
| | 1517 | Yalta, Crimea, podzol, USSR | 1951 |
| | 1077 | Brown soil taken from a cotton-field, Pekin | 1957 |
| | 2995 | Red earth soil near Sishan, Kun'min CPR | 1957 |
| 3 | 5001, 5002, 5014, 5211<br>5213, 5216, 5200, 5215<br>5009, 6912 | Red earth soil from rice field, Kun'min, CPR | 1957 |
| | 165, 155 | Light serozem, USSR | 1958 |
| | 467 | Medium loam, brown soil, USSR | 1959 |
| | 741 | Kandara, brown bog soil, USSR | 1959 |
| | 1511 | Yalta, Crimea, podzol, USSR | 1951 |
| | 2912 | Kola Peninsula, podzol, USSR | 1952 |
| | 478, 694 | Kandara, eroded bog soil, USSR | 1959 |
| 4 | 735 | Kandara, brown bog soil, USSR | 1959 |
| 5 | 380 | Brown soil taken from pine nursery in Pekin, CPR | 1957 |
| 6 | 1464, 1267, 1268 | Western Pamir, black brown soil, altitude 2320, USSR | 1959 |
| | 1238, 1242, 1234 | Western Pamir, mountain brown soil, USSR | 1959 |
| | 1521 | Western Pamir, high mountain soil, USSR | 1959 |
| | 1594, 1373 | Western Pamir, high mountain, primitive metal soil under snow, USSR | 1959 |

sporangia resemble short twigs and are not spiral (Fig. 1 A). Spores are oblong and cylindrical, their walls smooth and lacking thorns (Fig. 1 C). In medium I without glucose the cultures grow poorly, aerial mycelium is moderate in amount and pink in color, but forms sporangia well. They are long, branching, not spiral and infrequently bear 0.5-1 whorls at the end (Fig. 1 B). The spores are the same as in medium I with glucose.

In medium III [Krasil'nikov, 1950] growth is poor. Colonies are uneven, reddish or brownish-red. Aerial mycelia are lacking or very poorly developed in view of the rosy color. The medium is unstained. Sporangia are not formed.

In medium IV [Krasil'nikov, 1950] growth is good. Colonies are uneven, orange. Aerial mycelium is poorly developed and velvety. The medium is unstained. The sporangia are monopodial, elongate, branching, with incomplete terminal whorls (Fig. 1 B). Spores are oblong, cylindrical, smooth and lacking thorns (Fig. 1 C).

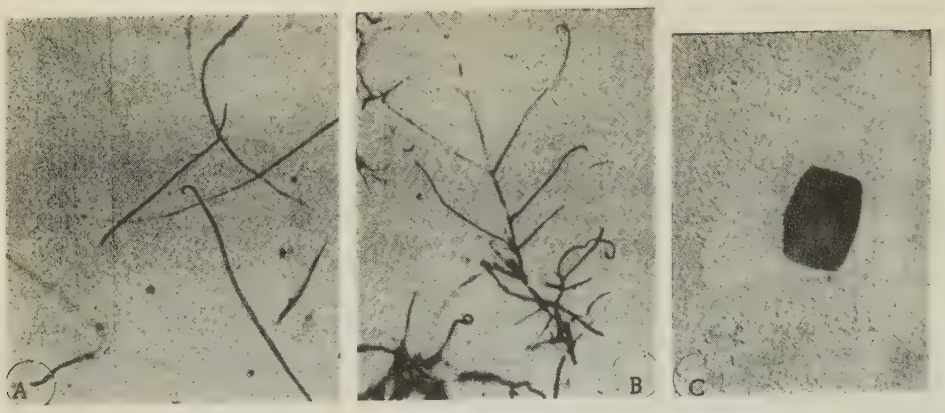


Fig. 1. Subgroup I (No. 3024). A) Sporangia in glucose-containing medium I at 12 days. 300×. B) The same in glucose-deficient medium. C) Spore under the electron microscope. 10,000×.

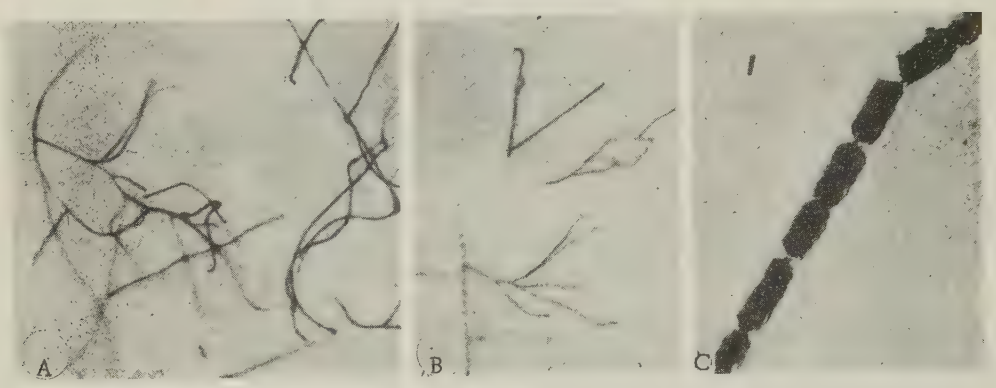


Fig. 2. Subgroup 2 (No. 5008). A, B) Sporangia in glucose-containing medium I at 12 days. 300 ×. C) Spores under electron miscrope. 6000 ×.

On potato agar the cultures grow very well. Colonies are uneven, flat and orange. Aerial mycelium is feeble and orange. The medium is unstained. Sporangia look like short twigs and are not spiral. Spores are oblong, cylindrical, smooth and lacking thorns (Fig. 1 C).

On starch-ammonium agar growth is good, colonies uneven, deep raspberry. Aerial mycelium is abundant, orange-pink to bright pink. The medium is unstained. Sporangia resemble short twigs and are straight. Spores are oblong, smooth and lacking thorns.

On wort agar growth is moderate to good. Colonies are flat and deep pink. Aerial mycelium is orange and velvety. The medium is unstained. Sporangia are not found.

In fish medium with glucose growth is good, colonies uneven, rust-orange. Aerial mycelium is lacking. The medium is unstained.

On meat-peptone agar growth is poor, colonies faintly plicate, pale red-brown. Aerial mycelium is lacking. The medium is unstained.

On potato slices growth is good. Colonies are plicate, orange. Aerial mycelium is abundant, orange. Sometimes an entire colony is covered with aerial mycelium. The potato slices are not stained brown.

On carrot slices there is no growth of cultures.

Subgroup 2. The second subgroup of orange actinomycetes consists of nine strains: Nos. 5008, 2933, 205, 1149, 1510, 1517, 5007, 5012, 5004.

In medium I the cultures grow well. Colonies are flat, orange. Aerial mycelium is abundant, orange or yellow-orange. The medium is unstained. Sporangia are arranged monopodially, and are elongate, tortuous but not spiral. Sometimes they arise from a branch in bundles (Fig. 2 A. B). Spores are bacilliform or oval, smooth and lack thorns (Fig. 2 C).

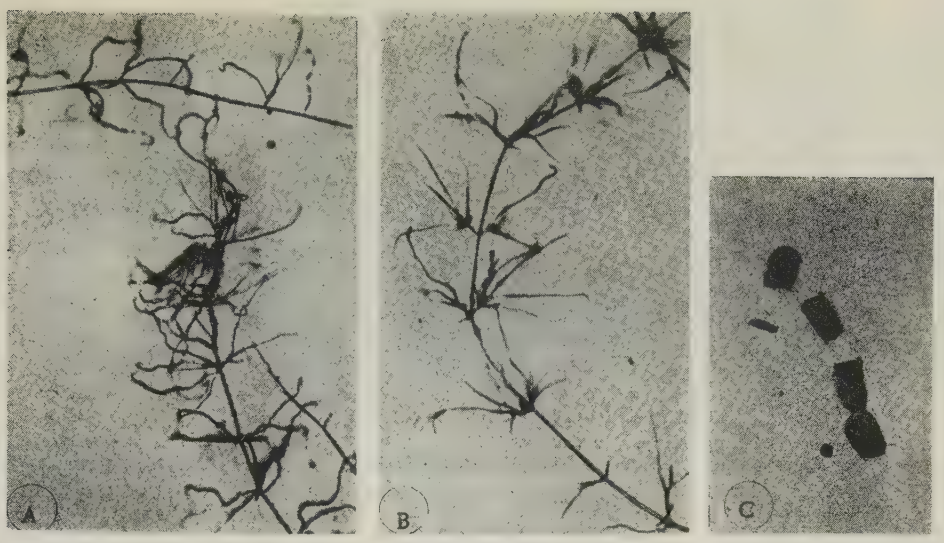

Fig. 3. Subgroup 3 (No. 1077). A, B) Sporangia in glucose-containing medium I at 12 days. 300 ×. C) Spore under electron microscope. 8000 ×.

In medium III growth is poor. Colonies are flat or faintly plicate and light brown. Aerial mycelium is lacking. The medium is unstained.

In medium IV growth is good. The colonies are flat, whitish or pale orange. Aerial mycelium is white and fluffy. The medium is unstained. Monopodial, elongate, tortuous but not spiral, separate oval sporangia form fascicles on the branches (Fig. 2 A, B). Spores are bacilliform or oblong, smooth and lack thorns (Fig. 2 C).

On potato agar the cultures grow well. Colonies are flat and orange. Aerial mycelium is well developed, pinkish and velvety. The medium is unstained. Sporangia are monopodial, long, twisting but not spiral. Spores are oval or oblong, smooth and lack thorns.

On starch-ammonium agar growth is good. Colonies are flat and rust colored. Aerial mycelium is pinkish and fluffy. The medium is unstained. Sporangia are monopodial, long, twisting, not spiral. Spores are oval or oblong and lack thorns.

On wort agar growth is moderate to good. Colonies are faintly plicate, flat and raspberry colored. Aerial mycelium is abundant and pink. The medium is unstained. The features of the sporangia and forms of spores are the same as in medium I with glucose.

In fish medium containing glucose growth is good. Colonies are flat or faintly plicate and orange. Aerial mycelium grows poorly and is orange or yellow-reddish. The medium is unstained. The features of spore-formation are the same as in medium I with glucose.

On meat-peptone agar growth is poor, colonies flat and pale orange. Aerial mycelium is lacking or it is feeble, weakly colored orangish. The medium is unstained. Sporangia are not found.

On potato slices growth is excellent. Colonies are plicate and pale orange. Aerial mycelium is well developed, pinkish-orange. The potato slices are stained a deep brown.

On carrot slices there is no growth of cultures.

Subgroup 3. In the third subgroup 20 strains of orange actinomycetes are included: Nos. 1077, 165, 478, 694, 155, 467, 741, 1511, 2912, 2995, 5001, 5002, 5014, 5211, 5213, 5216, 5200, 5215, 5009, 6912.

In medium I containing glucose the cultures grow well. Colonies are flat and orange. Aerial mycelium is pink, velvety or fluffy. The medium is unstained. Sporangia are distributed in whorls; at the end of long primaries and secondaries are formed incomplete whorls of 0.5-1.0 turns (Fig. 3 A, B). Spores are cylindrical, smooth and lack thorns (Fig. 3 C). In glucose-deficient medium I the sporangial features are the same as when glucose is present.

In medium III growth is poor. Colonies are flat or plicate, rusty-orange. Aerial mycelium is absent. The medium is unstained.

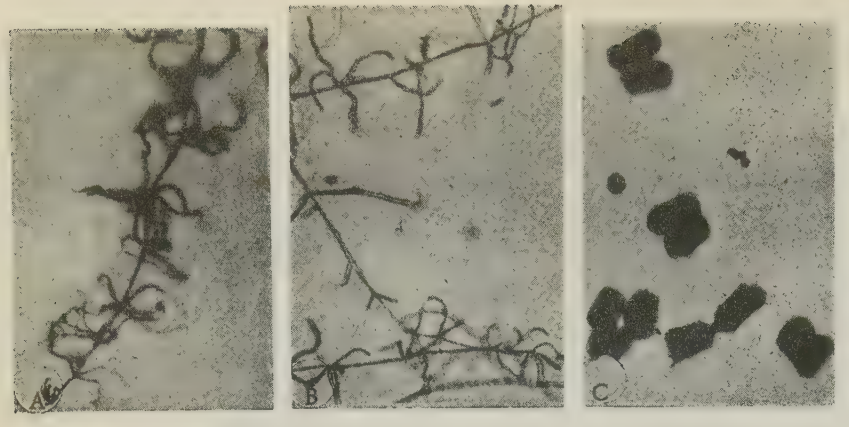

Fig. 4. Subgroup 4 (No. 735). A, B) Sporangia in medium I without glucose at 12 days. 300 X. C) Spores under the elctron microscope. 8000 X.

In medium IV the cultures grow well. Colonies are faintly plicate, bright orange or orange-red. Aerial mycelium is well developed, orange or pink and velvety. The medium is unstained. Sporangia and spores are the same as in medium I with glucose.

On potato agar the growth is good. Colonies are flat and orange. Aerial mycelium grows well, is pinkish and velvety. The medium is unstained. Sporangia are long, whorled, with primaries and secondaries (Fig. 3 A, B); spores are cylindrical, smooth and lack thorns (Fig. 3 C).

On starch-ammonium agar the growth is good, colonies are orange-red. The aerial mycelium is pink and velvety. The medium is unstained. Sporangia and spores are the same as in medium I with glucose.

On wort agar growth is good. Colonies are flat, orange-raspberry. Aerial mycelium is absent. The medium is unstained.

In fish medium with glucose the cultures grow well. Colonies are flat or plicate and orange. The aerial mycelium is poorly developed, yellow-orange, velvety,or it is absent.

On meat-peptone agar growth is poor. Colonies are flat, pale red. The aerial mycelium is absent. The medium is unstained.

On potato slices the cultures grow well. Colonies are plicate and bright orange. Aerial mycelium is abundant, pink to orange; the potato is stained brown.

On carrot slices there is no growth of cultures.

Subgroup 4. In this subgroup there is a single culture, No. 735.

In medium I the culture grows well. The colonies are flat, orange-red. Aerial mycelium is abundant and pink. The medium is unstained. Sporangia are gathered into twisting primary whorls and rarely into secondaries, sometimes as spirals with a single turn (Fig. 4 A, B). Spores are oblong, cylindrical, smooth and lack thorns (Fig. 4 C).

In medium III growth is poor. Colonies are flat or faintly plicate and light brown. Aerial mycelium is absent. The medium is unstained.

In medium IV the culture grows well. Colonies are flat, orange-red. The aerial mycelium is pinkish. The medium is unstained. Sporangia and spores are identical to those inmedium I with glucose.

On potato agar the culture grows well. The colonies are flat, orange. Aerial mycelium is pinkish and fluffy. The medium is unstained. Sporangia and spores are as in medium I.

Onstarch-ammonium agar the culture grows well. The colonies are flat and raspberry colored. Aerial mycelium is abundant, bright pink or pinkish. The medium is unstained; sporangia and spores are as above.

On wort agar the growth is good. Colonies are faintly plicate, brownish-red or raspberry colored. Aerial mycelium is very poorly developed and bright pink. Sporangia are not seen. The medium is unstained.

In fish medium with glucose growth is good. Colonies are flat and orange. The medium is unstained. Aerial mycelium is lacking or is poorly developed and cream colored. Sporangia are not observed.

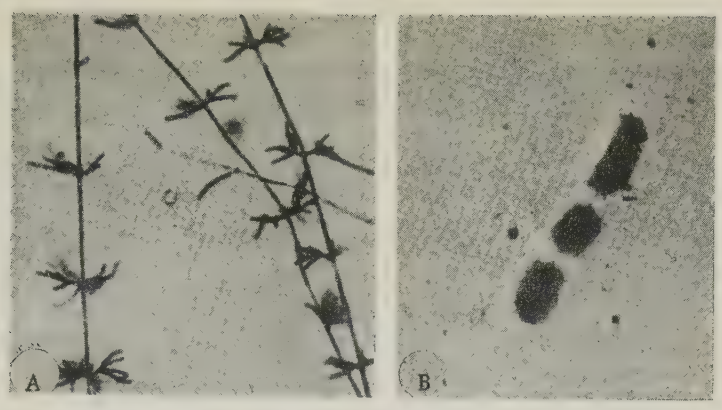


Fig. 5. Subgroup 5 (No. 380). A) Sporangia in medium I containing glucose at 12 days. 300 ×. B) Spores under the electron microscope. 8000 ×.

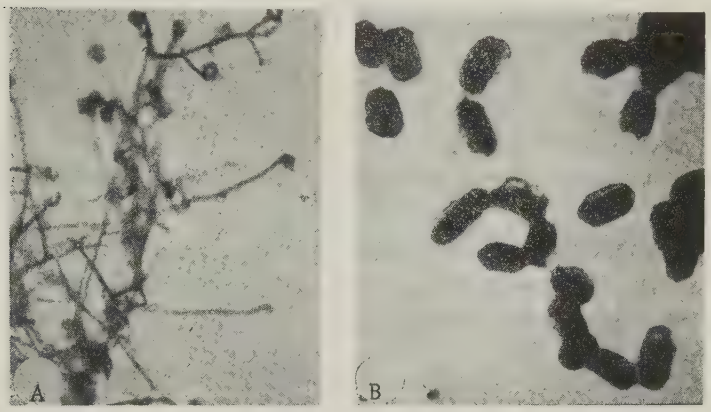


Fig. 6. Subgroup 6 (No. 1464). A) Sporangia in glucose-containing medium I at 12 days. 300 ×. B) Spores under the electron microscope. 8000 ×.

On meat-peptone agar growth is poor, the colonies flat and plicate. The aerial mycelium is absent. The medium is unstained.

On potato slices growth is good, colonies are orange. Aerial mycelium is orange or pink. The medium is unstained.

On carrot slices there is no growth of cultures.

Subgroup 5. In this subgroup only one strain, No. 380, has been placed.

In medium I containing glucose the culture grows well. The colonies are flat, raspberry or red-raspberry. Aerial mycelium is well developed, cream-pink and fluffy. The medium is unstained. Sporangia are short, straight, and form whorls. The whorls are primary and secondary with 3-5 sporangia in each (Fig. 5 A). The spores are long, cylindrical, smooth and lack thorns (Fig. 5 B).

In medium III the growth of the culture is good. Colonies are flat or faintly plicate and rust colored. Aerial mycelium is red-pink, fluffy. The medium is unstained; sporangia and spores are as above.

In medium IV the growth is excellent. The colonies are flat, red-orange. Aerial mycelium well developed, white and velvety. The medium is unstained. Sporangia and spores are as above.

On potato agar the growth is excellent. The colonies are flat, red-orange. Aerial mycelium creamy-pink and fluffy. The medium is unstained. The nature of the spores and spore formation is as above.

On starch-ammonium agar the growth is good. The colonies are flat, red-brown. Aerial mycelium is pale pink. The medium is unstained. Sporangia and spores are the same as above.

On wort agar the growth is moderate. Colonies are flat or faintly plicate. Aerial mycelium is absent or is poorly developed and pale pink. The medium is unstained.

In fish medium with glucose the growth is good. The colonies are flat and red-brown. Aerial mycelium is red-pink. The medium is stained brown. The sporangia and spores are the same as above.

On meat-peptone agar growth is good. The colonies are faintly plicate or flat and raspberry color. Aerial mycelium is absent. The medium is unstained.

On potato slices the growth is good. Colonies are raspberry color or red-orange. Aerial mycelium is orange-red and fluffy. Sporangia and spores are the same as above. The potato slices are stained deep brown.

On carrot slices there is no growth of cultures.

Subgroup 6. The remaining nine strains are placed here: Nos. 1464, 1267, 1238, 1242, 1234, 1268, 1373, 1521, 1594.

In medium I containing glucose the growth of the cultures is excellent. The colonies are flat, red-raspberry in color. Aerial mycelium grows well and is pink. The medium is unstained. Sporangia are monopodial, spiral, and form compressed or slightly extended whorls (Fig. 6 A). The spores are oblong and oval, smooth and lack thorns (Fig. 6 B).

In medium III growth is poor. The colonies are flat and colorless. Aerial mycelium is lacking. The medium is unstained.

In medium IV the growth is good. The colonies are flat, red-orange or raspberry. Aerial mycelium is well developed, pink and velvety. The medium is unstained. Sporangia are compressed or slightly extended spiral whorls distributed monopodially (Fig. 6 A). Spores are oblong, oval, smooth and lack thorns (Fig. 6 B).

On potato agar the growth of the cultures is good. The colonies are flat, rusty and orange. Aerial mycelium is abundant, pink. The medium is unstained. Sporangia and spores are as above.

On starch-ammonium agar growth is excellent. Colonies are flat, orange-red. Aerial mycelium is white to pink. The medium is unstained. Sporangia and spores are as above.

On wort agar the growth is moderate to good. Colonies are flat and deep brown. Aerial mycelium is absent. The medium is unstained.

In fish medium with glucose the growth is good. Colonies are flat and light raspberry. Aerial mycelium is lacking. The medium is unstained.

On meat-peptone agar the growth is poor, colonies colorless. Aerial mycelium is lacking. The medium is unstained.

On potato slices the growth of the cultures is excellent. The colonies are light raspberry in color. Aerial mycelium is poorly developed and pink. The potato is stained brown.

On carrot slices there is no growth of cultures.

## DIFFERENTIAL DIAGNOSIS

The morphological-cultural features of the orange actinomycetes are listed in Table 2.

Cultures of the first subgroup are distinguished by their bright orange color on cultivation in different nutrient media. The aerial mycelium grows poorly or is entirely lacking in the majority of nutrient media. Sporangia are straight, disposed monopodially, with 0.5-1.0 whorls. The spores are cylindrical, smooth and lack thorns.

The second subgroup stands close to the first, but in contrast to the latter shows good growth of a fluffy aerial mycelium. In all nutrient media the sporangia are not spiral but are long, twisting, monopodial and sometimes clustered in small bundles or panicles. Spores are bacilliform or oval, smooth and lack thorns.

TABLE 2

Cultural-Morphological Features of the Orange Actinomycetes of Different Subgroups

| Subgroup | Sporangia | Spores | amount of growth | Cultural characteristics in medium I | | extraction of pigment from the medium |
|---|---|---|---|---|---|---|
| | | | | colonies | aerial mycelium | |
| 1 | Monopodial, elongate, branching, not spiral, rarely with 0.5-1.0 whorl | Oblong, cylindrical, smooth, without thorns | Moderate | Uneven, bright orange | Poorly developed, orange, pink | No |
| 2 | Monopodial, elongate, twisting, not spiral, sometimes joined into bundles | Oblong, oval, smooth, without thorns | Good | Flat, orange | Abundant, fluffy, orange, orange-yellow | No |
| 3 | Long whorls, primary and secondary, forming incomplete terminal whorls of 0.5-1.0 turn | Cylindrical, without thorns | Good | Same | Velvety, pink | No |
| 4 | Primary whorls, rarely secondary, twisting, sometimes as spirals with a single whorl | Oblong, cylindrical, smooth, without thorns | Good | Flat, orange | Fluffy, pink | No |
| 5 | Short whorls, straight primary and secondary | Cylindrical, smooth without thorns | Good | Flat, red-raspberry, raspberry | Fluffy, creamy-pink | No |
| 6 | Monopodial, compressed or slightly extended spiral whorls | Oblong, smooth, without thorns | Good | Flat | Abundant, pink | No |

The third subgroup of orange actinomycetes is distinguished from the first and second by having good growth of aerial mycelium. Its sporangia are elongate primaries and secondaries. Whorls are formed, but not spirals, and at the ends of certain of these are incomplete whorls of 0.5-1.0 turn.

The fourth subgroup is not noticeably distinguished from the third by morphological-cultural characteristics. It is separated on biochemical and antimicrobial and other criteria which will be reported in a forthcoming article.

Cultures of the fifth subgroup, in contrast to previous subgroups, have very short primary and secondary whorls. The colony coloration is red-orange, raspberry-red or raspberry.

The sixth subgroup is characterized by flat, compressed or slightly extended spiral whorls. Sporangia resemble little fists on long branches. These sporangia are arranged rather densely on usually monopodial branches.

This work was carried out under the guidance of Corresponding Member of The Academy of Sciences, USSR, Professor N. A. Krasil'nikov and A. I. Korenyako, to whom the author expresses his deep appreciation. The author expresses his gratitude to R. M. Legunkova for assistance with the electron microscopy of the cultures.

## SUMMARY

1. A study has been carried out of 62 cultures of orange actinomycetes isolated from various soil samples of the Chinese People's Republic and of the Soviet Union.

2. These cultures have been divided into 6 subgroups according to their morphological-cultural characteristics.

3. Six species have been isolated differing in their cultural-morphological peculiarities as well as in physiological and antibiotic characters and in the specificity of antagonism (this will be reported in a forthcoming paper).

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**All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.**

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# EFFECT OF VARIOUS NITROGEN SOURCES UPON THE SURFACE OF THE SPORE ENVELOPE OF ACTINOMYCETES

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The possibility of utilization of appearance of surface of spore envelope as a diagnostic criterion for classification of actinomycetes was confirmed by studies of several investigators (Kuster, 1953; Corbaz, Ettlinger and Hütter, 1958; Preobrazhenskaya et al., 1959).

However, the stability of this character was not investigated intensively under different conditions of cultivation (particularly under different nutritional conditions). Only occasional data are available in the literature pertaining to effect of various nitrogen sources in a nutrient medium on formation of growth on spore envelope of actinomycetes.

Thus Flaig, et al. (1955), observed that in cultures grown in nutrient medium containing inorganic source of nitrogen there is a less pronounced development of spiny spores than when grown in media containing organic nitrogen. Different results were obtained by Lesheval'e and Tikhonenko (1960). Of the 29 nutrient media studied by them the best formation of spiny actinomycete spores proved to be in media containing nitrates (particularly media with $NaNO_3$ and starch).

The present work is devoted to determination of the effect of various nitrogen sources in nutrient media on shape and dimensions of growth on spore surface of actinomycetes.

## METHODS

Pridham and Gottlieb's medium (1948) of the following composition was utilized in this experiment (g): $KH_2PO_4$-2.38; $K_2HPO_4$-5.65; $MgSO_4 \cdot 7H_2O$-1.0; $CuSO_4 \cdot 5H_2O$-0.064; $FeSO_4 \cdot 7H_2O$-0.011; $MgCl_2 \cdot 4H_2O$-0.079; $ZnSO_4 \cdot 7H_2O$-0.0015; agar-30.0; distilled water-1 liter; pH 6.8-7.0. Glucose (1.5%) was added to the medium as the source of carbon. The medium was divided into aliquots, to which were added different sources of nitrogen: alanine, valine, glycine, pehnylalanine, lysine, cystine, aspartic acid, glutamic acid, norleucine, tyrosine, methionine, proline, $NaNO_3$, $(NH_4)_2SO_4$. Medium free of nitrogenous compounds served as control. All the enumerated sources of nitrogen were added to the medium in quantities to form 28 mg % nitrogen. Prepared media were distributed into test tubes and sterilized twice in an autoclave for 30 min at 0.5 atm. Suspensions of spores of the following species of actinomycetes, washed in physiological saline and centrifuged, were deposited on agar slants: Actinomyces sp. (Nos. 1943 and 7551), with short hairy spores; Actinomyces variabilis var. roseus (Nos. 8820 and 2366), with long hairy spores; Actinomyces griseorubens (Nos. 1581 and 15403), with medium spines; Actinomyces sp. (Nos. 6124 and 3124), with small spines; and Actinomyces sp. (Nos. 5434 and 16725) with long spines. Cultures used for inoculation were grown on mineral medium with starch (Gauze, et al., 1957).

Inoculated test tubes were kept for 2-3 weeks at 28 C. Samples for microscopic examination were prepared in the usual manner, as impressions on colloidion films. Actinomycete spores were examined by means of an electron microscope ÉM-3, magnified 4000 times.

Appearance of spores, the nature and dimensions of growth of spore surface were determined.

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TABLE 1

Formation of Aerial Mycelium and Spores on Media with Different Nitrogen Sources

| Nitrogen source | Act. sp. 7551 | | Act. sp. 1943 | | Act. variabilis var. roseolus 8820 | | Act. variabilis var. roseolus 2366 | | Act. coerulescens 1581 | | Act. coerulescens 5403 | | Act. griseorubens 6124 | | Act. griseorubens 3124 | | Act. sp. 5734 | | Act. sp. 16725 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | aerial mycelium | spores | aerial mycelium | spores | aerial mycelium | spores | aerial mycelium | spores | aerial mycelium | spores | aerial mycelium | spores | aerial mycelium | spores | aerial mycelium | spores | aerial mycelium | spores | aerial mycelium | spores |
| Alanine | + | + | + | + | + | + | + | + | + | + | + | + | ± | + | ± | + | + | + | + | + |
| Valine | + | + | + | + | ± | + | ± | + | + | + | + | + | ± | + | ± | + | + | + | + | ± |
| Glycine | + | + | + | + | + | + | + | + | + | + | + | + | ± | + | ± | + | + | + | + | ± |
| Phenylalanine | + | + | ± | + | ± | ± | ± | + | + | + | + | + | ± | ± | ± | ± | + | + | + | ± |
| Lysine | + | + | + | + | + | ± | + | + | + | + | + | + | ± | ± | ± | ± | + | + | + | ± |
| Cystine | + | + | + | + | ± | ± | ± | + | + | + | + | ± | ± | ± | ± | ± | + | + | + | ± |
| Glutamic acid | + | + | + | + | ± | ± | ± | + | + | + | + | + | ± | ± | ± | ± | + | + | + | ± |
| Norleucine | ± | + | + | + | ± | ± | ± | + | ± | + | + | + | ± | ± | ± | ± | + | + | + | ± |
| Tyrosine | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Methionine | + | + | + | + | + | + | + | + | + | + | + | + | ± | ± | ± | ± | + | + | + | + |
| Proline | + | + | + | + | + | + | + | + | + | + | + | + | ± | ± | ± | ± | + | + | + | + |
| $NaNO_3$ | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| $(NH_4)_2SO_4$ | ± | + | ± | + | − | − | − | − | ± | − | + | + | − | − | − | − | ± | + | ± | − |
| Control, no N | + | − | + | + | − | − | − | − | + | + | + | + | + | − | − | − | ± | − | − | − |

Legend: +) Good and adequate growth of aerial mycelium and presence of spores ±) weak growth of aerial mycelium; −) absence of aerial mycelium and spores.

TABLE 2

Stability of Appearance of Growth on Spore Surface of Actinomycetes Grown on Media with Different Nitrogen Sources

| Nitrogen source | Act. sp. № 1943 | | | Act. sp. № 7551 | | | Act. variabilis var. roseolus № 8820 | | | Act. variabilis var. roseolus № 2366 | | | Act. cerulescens № 1571 | | | Act. cerulescens № 5403 | | | Act. griseorubens № 6124 | | | Act. griseorubens № 3124 | | | Act. sp. № 5734 | | | Act. sp. № 16725 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | surface of spore envelope | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | spiny | hairy | smooth | spiny | hairy | smooth | spiny | hairy | smooth | spiny | hairy | smooth | spiny | hairy | smooth | spiny | hairy | smooth | spiny | hairy | smooth | spiny | hairy | smooth | spiny | hairy | smooth | spiny | hairy | smooth |
| Alanine | — | + | + | — | + | + | — | + | — | — | + | — | + | — | + | + | — | + | + | — | — | + | — | — | + | — | + | + | — | — |
| Valine | — | + | + | — | + | + | no spores | | | no spores | | | + | — | — | + | — | — | no spores | | | | | | + | — | + | + | — | + |
| Glycine | — | + | + | — | + | + | — | + | + | — | + | + | + | — | + | + | — | — | + | — | — | + | — | — | + | — | + | no spores | | |
| Phenylalanine | — | + | — | — | + | + | no spores | | | — | + | + | + | — | + | + | — | + | no spores | | | | | | + | — | + | no spores | | |
| Lysine | — | + | + | — | + | + | no spores | | | no spores | | | + | — | + | + | — | + | no spores | | | | | | + | — | + | » | » | |
| Cystine | — | + | — | — | + | + | | | | — | + | — | + | — | + | + | — | — | » | » | | | | | + | — | + | + | — | — |
| Glutamic acid | no spores | | — | + | + | — | | | | no spores | | | no spores | | | no spores | | | » | » | | | | | no spores | | | no spores | | |
| Norleucine | — | — | + | — | + | — | » | » | | no spores | | | + | — | — | + | — | | » | » | | | | | + | — | — | + | — | — |
| Tyrosine | — | + | — | — | + | — | — | + | + | — | + | — | + | — | — | + | — | — | + | — | — | + | — | — | + | — | — | + | — | + |
| Methionine | — | + | + | — | + | + | — | + | + | — | + | — | + | — | + | + | — | — | no spores | | | | | | + | — | — | no spores | | |
| Proline | — | + | + | — | + | + | — | + | — | — | + | — | + | — | — | + | — | — | no spores | | | | | | + | — | — | + | — | + |
| $NaNO_3$ | — | + | + | — | + | — | — | + | — | — | + | — | + | — | + | + | — | + | + | — | + | + | — | + | + | — | + | + | — | + |
| $(NH_4)_2SO_4$ | — | + | + | — | + | — | no spores | | | no spores | | | no spores | | | + | — | — | no spores | | | | | | + | — | — | no spores | | |
| Control (without nitrogen) | — | — | — | no spores | | | no spores | | | no spores | | | + | — | — | + | — | — | | | | | | | no spores | | | no spores | | |

Legend: +) Presence of spore growth; —) absence of spore growth.

## RESULTS

As can be seen from data in Table 1, best growth of aerial mycelium and formation of spores in all cultures under investigation was observed on media with $NaNO_3$, alanine and tyrosine. Media containing lysine, norleucine and $(NH_4)_2SO_4$ were intermediate in nature. The least suitable medium for formation of aerial mycelium was that containing glutamic acid (only 3 out of 12 strains showed growth of aerial mycelium and only one of these formed spores). The obtained results confirmed Lesheval'e's data, according to which media containing glutamic acid and ammonium sulfate were worse for sporulation than media with nitrates.

Development of actinomycetes on media with different sources of nitrogen did not bring about any changes in the shape of spores. Therefore,we are not presenting the data from studying spore shape by means of electron microscopy.

Results of experiments dealing with effectsof different nitrogen sources on formation of surface growth of spores of actinomycetes are presented in Table 2.

As can be seen from the data in this table, the nature of growth on spore surface of five investigated species is characteristic of these species and remains constant in their development on media with different nitrogen sources.

Species with spiny spores did not acquire, in even a single instance, hairy growth, and species with hairy spores did not become spiny. All variations of both types did not lose their outgrowths. However, considerable variation was observed in the size of spines and hairs. This applies, first of all, to species A. variabilis var. roseus (with long hairs) and Actinomyces sp. 5434 and 16725 (with long spines). In most media the spore surface in the strains of these species was covered with hairs and spines of quite different length. Individual spores were encountered completely devoid of growth.

In strains with spore surfaces with medium size growth, smooth spores and spores with short spiny and hairy growth were observed under almost all experimental conditions. Greatest consistency was observed in strains of species with short growth on spore surfaces (in Actinomyces sp. Nos. 1943 and 7551 and in A. griseorubens). Spores with medium growth were seldom encountered in this case, while spores with long growth were never observed.

Smooth spores were present in almost all cases, in addition to spores with short spines or hairs.

By comparing the effect of various nitrogen sources on development of growth on spore surfaces, it is possible toconclude that the characteristic growth on the surface forms mostly when the nitrogen source is favorable for development of aerial mycelium and spores (in the variants with $NaNO_3$, alanine and tyrosine).

## SUMMARY

1. A study was made of the effect of various amino acids and mineral nitrogen sources on formation of growth on spore surface of 12 strains of actinomycetes belonging to the species and varieties: Actinomyces variabilis var. roseus, Actinomyces coerulescens, Actinomyces griseorubens and Actinomyces sp.

2. Media with $NaNO_3$, alanine, and tyrosine proved to be most favorable for formation of spore-bearing aerial mycelium and for development of growth on spore surface. Least favorable was the medium with glutamic acid.

3. The nature of growth on spore surface (spines, hairs) is constant and independent of nitrogen sources in nutrient media.

4. Dimensions of growth on spore surface in most of the investigated strains remained constant under all experimental conditions. Significant variation in size of sporogenous growth was observed only in strains with long spines and hairs (strains 8820, 2366, 5734, and 16725).

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

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# MOVEMENT OF BACTERIAL COLONIES

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In some types of bacteria the colonies show active displacement over the surface of agar media.

Information on this subject has been published by the following: Müller (1912);Roberts (1935); Rozhdestvenskii and Medvinskaya (1938); Russ-Münzer (1938); Smith and Clark (1938); Knaysi (1951); Murray and Elder (1949); Shinn (1938, 1940); Chistovich (1946). A detailed survey of these works was made by Săsarmăn (1957).

The above listed investigators have established the ability for active displacement in colonies of Bacillus alvei, Bacillus circulans, Bacillus rotans, Bacillus migrans and others. They observed the ability for translational and rotational movement Rozhdestvenskii and Medvinskaya, 1938; Müller, 1912; Murray, Elder, 1949; Roberts, 1935; Russ-Münzer, 1938; Shinn, 1938, 1940). The colony assumes various shapes, depending on the nature of the movement—the shape may be welt-like, ring-like, snail-like, bullet-like or comet-shaped.

The majority of scientists hypothesize that colony movement depends on the synchronized movement of flagella, but the reasons for this have not yet been established. It was noted that colony movement is independent of percentage agar content (Roberts, 1935) and is observed in the absence of a visible water film on the substrate surface (Knaysi, 1951). The goal of the present experiment was to clarify the mechanism of and the reasons for colony movements on media surfaces.

## EXPERIMENTAL

The experimental subjects were twelve strains of Bacillus coloniomobile—a microbe described by us in a previous work (Sherstoboev, 1958). The colony displacement was studied on media with varying content of nutrional substances, agar and sodium chloride, but most of the observations were made with cultures grown on the usual meat-peptone agar (MPA), containing 2% agar and 0.5% sodium chloride. The inoculations, as a rule, were made by a stab into the center of a Petri dish. This enabled us to observe the spread of the culture and the colony movement over a period of many days.

The nature of colony movement. Colonies of the investigated microorganism are capable of translational, rotational and combined movements.

The translational motion is characteristic for the bullet-like and the welt-shaped colonies. A mass of bullet-like colonies was observed during the first two days; as the culture aged their number was noticeably decreased; in many cases, however, even in ten day old cultures, an occasional colony of this type could be found.

The speed of motion of the bullet-like colonies was determined with a phase contrast microscope, aided by a micrometer scale and a stopwatch. The usual distance measured was a 110$\mu$ course. Table 1 shows displacement speeds in relation to the age of the culture.

The same colony, measured at very close time intervals, varied in the speed of its movement from 11.8$\mu$ per min to 68$\mu$, with an average speed of 33.7± 4.5$\mu$.

During translational movement we observed the displacement of the bacterial mass within the bullet-like colonies.

TABLE 1

Locomotion Speed of Bullet-Like Colonies in relation to Culture Age

| Culture age, days | Speed of movement, μ/sec | | |
|---|---|---|---|
| | maximum | minimum | average |
| 1 | 4.27 | 1.8 | 3.04 |
| 2 | 2.68 | 1.46 | 2.32 |
| 3 | 1.86 | 1.11 | 1.73 |
| 4 | 1.24 | 0.78 | 1.21 |
| 5 | 0.86 | 0.49 | 0.80 |
| 6 | 0.77 | 0.43 | 0.71 |
| 7 | 0.62 | 0.37 | 0.58 |
| 8 | 0.53 | 0.013 | 0.37 |
| 9 | 0.37 | 0.01 | 0.23 |
| 10 | 0.05 | 0.005 | 0.03 |

TABLE 2

Rotation Rate of Rotating Colonies

| Culture age, days | Rotation rate ω, rad/sec | | |
|---|---|---|---|
| | minimum | maximum | average |
| 1 | 0.061 | 0.523 | 0.321 |
| 4 | 0.054 | 0.117 | 0.087 |
| 7 | 0.049 | 0.059 | 0.058 |
| 10 | 0.019 | 0.047 | 0.029 |

Rotation in its pure form was rarely observed, and occurred mainly when the colony was closely surrounded by bacterial growth. Rotational movement was observed in the smaller colonies with two- or three-layer structures. The top layer of bacterial cells rotated over a wider surface than the first tier of cells. Consequently, in these cases the base for rotation was the bacterial layer of the first tier, as the round colonies rotated, sliding over the nutrient medium surface.

With rotational movement the colonies sometimes assumed a ring-like form, in the center of which was a promontory of a snail-like shape (Fig. 1). In other cases the rotating colony itself resembled a snail's shell (Fig. 2). Sometimes, ring-like shapes with a cell-free center were formed, not as the result of rotation, but by other means to be described later.

The rotation speed of the colonies varies and depends on various factors—among these the age of the culture. We determined the rotation rate (angular velocity), $\omega$, under a phase contrast microscope. Employing a stopwatch, we determined the time required for the colony to rotate through 90°, and then the rotation rate was expressed in radians per second.

It is interesting to note that the colony rotation speed, according to Rozhdestvenskii and Medvinskaya (1938) was 0.00087 rad/sec, while according to Shinn (1938) it was 0.0026 - 0.00093 (we converted it to radians). Thus, the rotation of B. coloniomobile colonies exceeded previously known speeds by 20-200 times.

Murray and Elder (1949) found that the colonies of B. circulans, B. alvei and B. sphericus var. rotans rotated mainly in an anticlockwise direction (66%), while only 34% rotated in the opposite direction. According to Shinn (1940), out of 300 colonies there were only two rotating clockwise.

We determined the rotation direction of 3690 colonies of B. coloniomobile and found that the numbers rotating in both directions were approximately equal: 50.25% rotated clockwise and 49.75% anticlockwise.

Combined transitional and rotational movement is encountered more frequently than the first two kinds. We frequently observed a translational—rotational motion which left a trace in the form of two parallel ridges of bacterial cells. Between the ridges there were many arc-like rotational traces, the distance between which, i.e., the "stride" of the colony, varied between 3—40.8 μ, with an average of 11.7 ± 0.26 μ. This method of movement is characteristic of the typical round colonies, "traveling" singly or in groups.

We twice observed the formation of figures reminiscent of a trilobite imprint. Their formation is connected with destructive changes taking place in the gel at a depth of several microns from the medium surface. The microbe mass, reaching a zone of destruction, circumvented it, forming a trilobite figure (Fig. 3). This formation took place on meat-peptone agar of the usual composition.

On rare occasions one could observe how the usual colony started stretching into a sausage-like figure, directing itself toward a free surface of the medium. Its motion frequently terminated in a circular displacement with its ends locking to form a ring-like colony with a cell-free center.

The spread of the culture over the medium occurred not only as the result of the colony displacement, but also by other means. One of these methods is proliferating growth. It is characteristic for colonies growing on media containing 2.5% agar or more, and on media that are drying out. In this latter case, in the center of the Petri dish we observe a typical growth, while the periphery of the dish shows proliferating growth. Such a culture type is seen in Fig. 4.

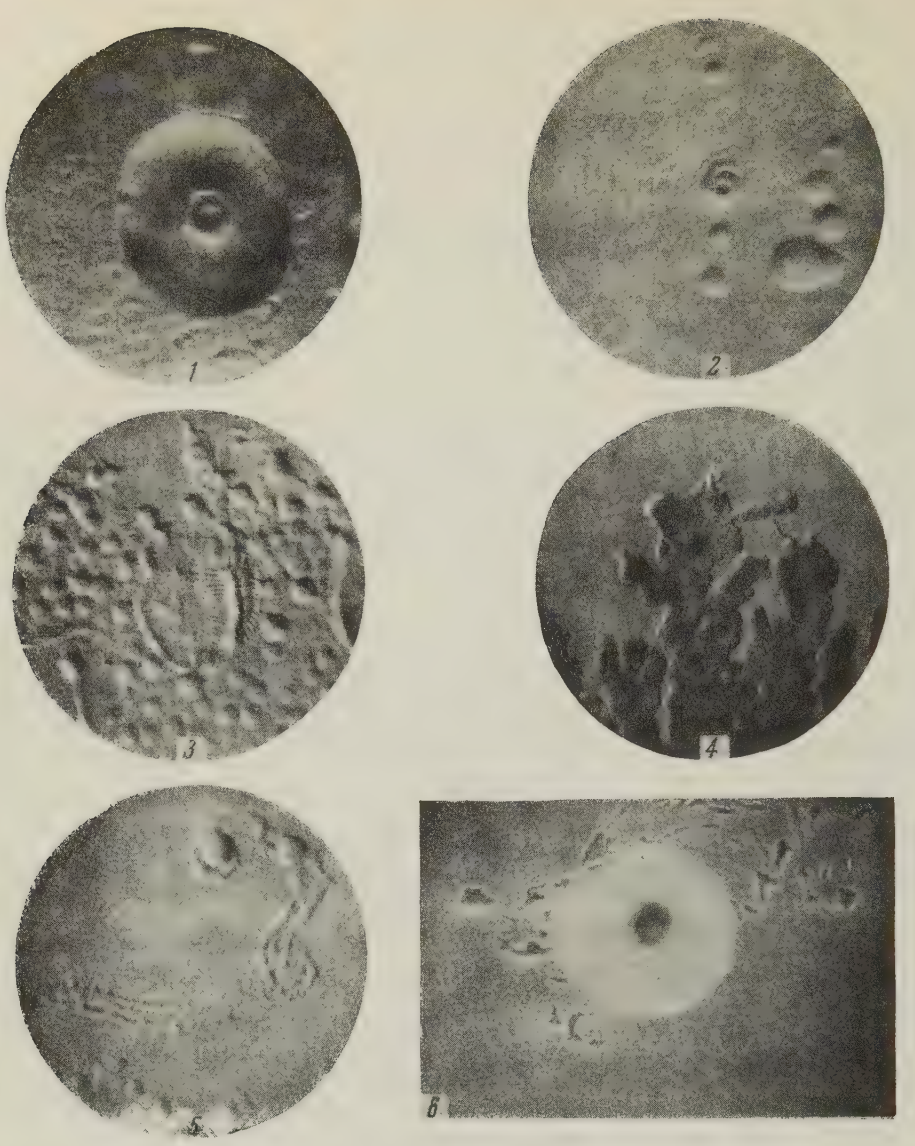

Fig. 1. Ring-shaped colony of B. coloniomobile with a snail-like center. 50 ×.
Fig. 2. Snail-shaped colony. 50 ×.
Fig. 3. Trilobite-like form, surrounded by typical colonies of B. coloniomobile. 50 ×.
Fig. 4. Proliferative growth of culture on 2.5% agar. 50 ×.
Fig. 5. Three-ridge trace of a displacing colony. 50 ×.
Fig. 6. Complicated trace left by a displaced thread-like form of B. coloniomobile. 660 ×.

Sometimes we observed the travel of whole "continents" of cells; the size of these formations reached 0.5×0.5–1.3×0.9 cm. They were characterized by translational motion with a tendency toward slight rotation.

On a fresh nutrient medium the microorganisms multiplied and more or less diffusely spread over a significant area. This process was independent of active cell movement, but rather took place due to surface tension of the medium's liquid phase and to convection.

The dispersed single cells then aggregated into small groups, during which time it was seen that the cells moved while lying in positions transverse to the direction of displacement, which indicated the passive nature of this displacement. The resultant conglomerations of cells gradually merged into larger aggregates, out of which the typical colonies of this strain were formed.

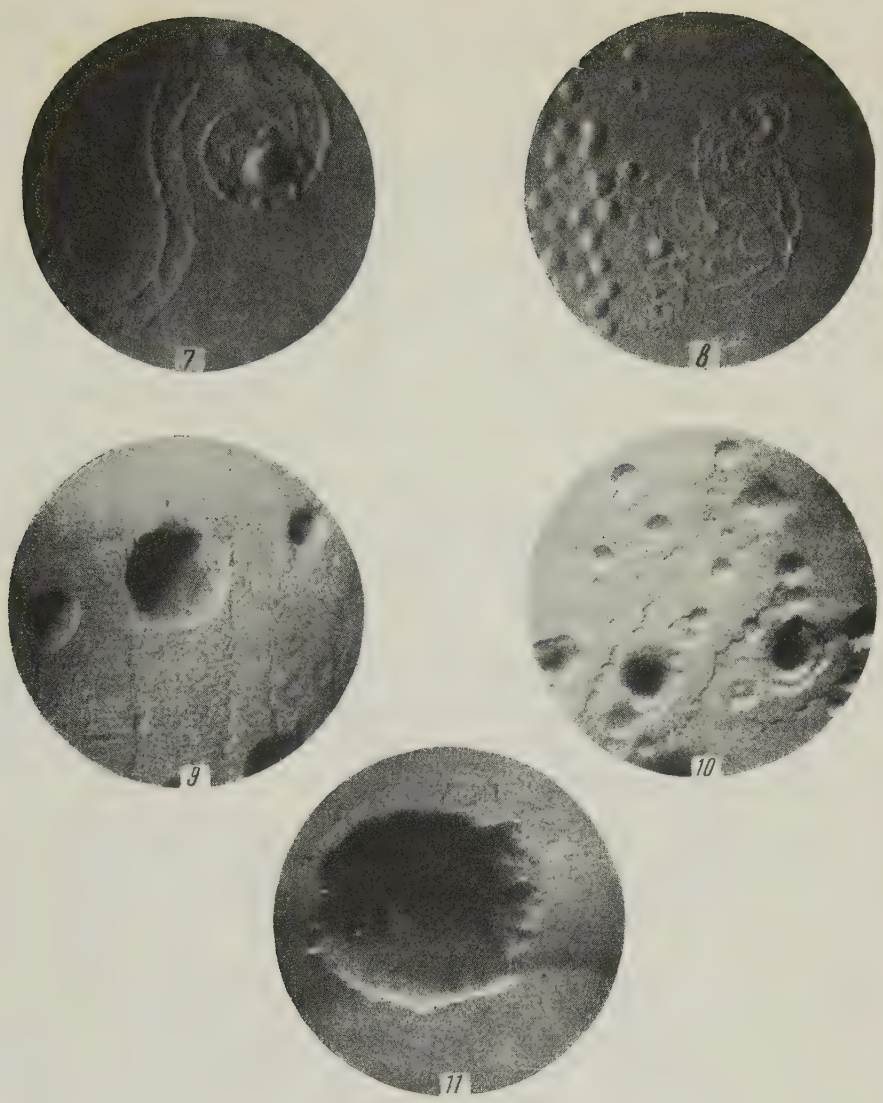

Fig. 7. Colony trace resembling a mirror-image question mark. 50 ×.
Fig. 8. Intricate looped trace of a colony. The colony has spread along the trace. 50 ×.
Fig. 9. Traces of parallel-moving colonies. 50 ×.
Fig. 10. Traces of colonies moving in a fan shape. 50 ×.
Fig. 11. Colony of irregular shape growing on the upper part of an agar slant. 70 ×.

Between the cell islands we frequently observed thread-like forms reaching lengths of 72 $\mu$. The long thread-like processes actively moved over the substrate—in straight lines, in arcs, in waves or in circles. Their linear displacement was of a low speed (0.33 to 38.4 $\mu$ per min); the circular motion, however, was very rapid: $\omega = 0.0269-0.367$ radians per sec, with an average $\omega = 0.077$ radians per sec—i.e., the circular motion of the thread-like forms and the rotation of colonies had the same speed.

Traces of displaced colonies. Moving colonies frequently leave traces on the medium in the form of two parallel ridges, consisting exclusively of vegetative cells. Sometimes the colonies undergoing displacement have in their centers the beginnings of spore formation. The traces of such colonies too, however, consist only of vegetative cells.

Therefore, traces are formed with comparatively young cells capable of vegetative growth and frequently giving rise to secondary colonies. With time all the cells in the traces may form spores, but this is already a secondary phenomenon.

We sometimes observed the formation of a trace consisting of three ridges of bacterial cells (Fig. 5), whereas in other cases the colonies frequently left but one ridge on the medium, for example the bullet-like colonies upon turning.

Frequently between the trace ridges there was a space free of cells; in other cases this space contained a mass of cells lying close to each other. In these cases the cells from the traces merged into more compact groupings, giving rise to secondary colonies.

In some cases the movement of colonies over the medium did not leave a cellular trace; the phase contrast microscope, however, revealed a clear trace consisting of a homogeneous bacterial mucoid with the water it had absorbed from the medium. Homologous traces were sometimes left by bullet-like colonies and by the thread-like forms (Fig. 6).

Infrequently, the displacing colonies left on the medium quite a complicated trace, resembling a question mark or its mirror image (Fig. 7), or else intricately woven loops (Fig. 8).

The beginning of the trace in singly moving colonies was nearly always tangentially directed with respect to the line of bacterial growth. In most cases the width of the trace was more or less the same. Colonies moving en masse left parallel traces which had the same width throughout their length (Fig. 9). Apparently this was due to the fact that the diameter of the colony did not change, as the number of cells left behind on the medium equalled the number of newly formed microbes.

With the fan-like displacement of a colony the beginning part of the trace was significantly narrower than the end (Fig. 10). The formation of a widening trace is explainable by the fact that during fan-like motion the colonies were in a position favorable for the flow of nutrient substances toward them. With this type of displacement the newly formed daughter colonies showed tendencies toward movement tangential to the trace of the mother colony, and thus the trace developed dichotomously.

Thread-like forms of bacteria. On a free medium surface adjacent to bacterial growth, one often observes long thread-like forms reaching 33-72$\mu$ in length. On media that are drying out these thread-like forms give rise to the proliferative growth of the culture, since the threads are overgrown on both sides by regular cells. This is what causes the tongue-like processes on the peripheries of the colonies.

In other cases the thread-like forms are capable of independent displacement over the surface of a medium. Their path may be linear, arc-shaped, wave-like or loop-like. In our experiments the speed of their displacement varied from 0.33 to 15 $\mu$ per min, the average speed being $7.7 \pm 0.75$ $\mu$. The movement of thread-like cells often ended in a circular motion, with a diameter of 50-60 $\mu$. When the thread was sufficiently long its ends sometimes joined, forming a ring. Sometimes we noted a dissolution of the thread-like cell—the process was completed within 20-30 min. The reasons for this phenomenon have not yet been determined.

The movement of the thread-like cells was not of a uniform nature. Often the threads were in a state of repose for a long period of time, and then suddenly started displacement. After a short period of activity a quiescent state again ensued.

Quite often the thread-like forms comprised the direct periphery of bullet-like colonies.

Composition of the medium as an influence on movement. Contrary to the opinion of Roberts (1935), who considered that the percentage agar content of the medium did not influence colony movement, we found that the highest activity occurred on media containing 1.5-2% agar. With an agar content of 0.5-1.0% the culture lost its colony-forming ability and grew in the spreading fashion of Proteus vulgaris. On media with 2.5-3% agar there occurred colonies of irregular form, with a very slow translational movement (up to 0.08 $\mu$ per sec), and the general appearance of the culture differed sharply from cultures grown on 1.5-2% agar (Fig. 11).

Likewise, colonies of irregular form occurred as the substrate dried out. Changes in the round type of colony started with the formation on its periphery of thread-like outgrowths, overgrown by other cells and resembling tongue-like structures.

The thickness of the nutrient substrate layer has a material influence on colony movement. When grown in Petri dishes containing agar layers of 1.5, 3 and 4.5 mm in thickness, the cultures developed on the second day after innoculation; and whereas on the thin layers the colonies were already spread profusely over the medium's surface, on the thicker layers such a spread did not begin until the fifth or sixth day. By ten days the cultures on

TABLE 3

Spread of Culture over Medium Surface in relation to Sodium Chloride Concentration

| Culture age, hr | Concentration of NaCl in the medium, % | | | | |
|---|---|---|---|---|---|
| | 0.4 | 0.5 | 0.7 | 0.9 | 1.0 |
| 24 | 5 | 11 | 4 | 3 | 1 |
| 48 | 13 | 22 | 14 | 7 | 2 |
| 72 | 32 | 60 | 21 | 9 | 4 |
| 96 | 45 | 72 | 34 | 11 | 10 |
| 120 | 57 | 75 | 41 | 16 | 14 |
| 144 | 66 | 75 | 48 | 19 | 19 |
| 168 | 75 | 75 | 53 | 25 | 22 |

all Petri dishes had spread to the same extent. This experiment showed that colony movement depends in part on the abundance or insufficiency of nutrient substances, and also, apparently, on the establishment of diffusion forces between the parts of the medium with growth and the parts free of growth. The sooner the nutritive substances are exhausted, the sooner the colonies spread over the substrate.

In a previous article (Sherstoboev, 1958) we noted the influence of sodium chloride concentration on the motility of individual cells of B. coloniomobile. In the current work we are presenting data on the influence of this substance upon colony movement. By using meat-peptone agar with varying concentrations of sodium chloride, we established that colonies growing on media with 0.4-0.5% NaCl possess the highest degree of activity. When the concentration of NaCl was raised the displacement speed of the colonies was decreased.

Mechanism of colony movement. The translational movement or forward movement of round colonies begins with a wave-like displacement of the bacterial mass to the frontal zone. Consequently, the rear end of the colony becomes thinner. The cell mass flowing into the frontal part forms currents similar to those observed in the freezing of ice layers. The accumulated mass gradually flows onto a free medium surface. By this method colonies shift slowly, not more than 10μ an hr.

During translational motion of the bullet-like colonies one can likewise notice in them the displacement of bacterial cells. It takes place from the rear part to the front and is achieved with considerably greater speed than in the round colonies.

The positioning of cells inside the bullet-like colonies is frequently disorderly; the majority of cells, however, do lie in the direction of the column. Not infrequently, on the periphery of such colonies we find long, thread-like cells curved into the shape of the colony's edge.

Rotational movement begins with a one-sided displacement of the mass inside the colony. In one of the peripheral regions of the colony a bacterial mass accumulates which noticeably raises itself over the other regions. Then the bacterial cells "flow down" to lower regions, thus providing the "push" for the rotation. At first, movement occurs in the peripheral belt, and then gradually the more central parts of the colony become involved. In other cases, the rotation starts with the formation in one sector of the colony of small radial folds, which shift in the direction of the impending rotation.

Watching moving colonies under the phase contrast microscope, one can sometimes observe that the frontal part of the colony drives in front of itself a film of water in the form of a ball.

The fluidity of the bacterial mass depends on the ability of the bacterial cells to attract significant amounts of moisture from the medium, and on their secretion of hydrophilic metabolites of a mucous nature. This was confirmed by the observations that a moving colony drives before it a ridge of mucous and moisture and that the movements of round, comet-like colonies and thread-like forms sometimes leave a cell-free trace easily observed with the phase contrast microscope. Such traces persist on the medium for great lengths of time and do not spread over its surface. Finally, when a moving colony encounters colonies of other types, the bacterial mass of the moving colony flows around these with extreme speed. Upon termination of this process, the periphery of the heterogenous colony is surrounded by a zone of cells passively swimming in a completely transparent fluid; also,

upon the diffuse dispersion of B. coloniomobile cells over the surface of the medium (if this takes place near a nonmotile strain, e.g., Micrococcus), the cells of the nonmotile strain likewise become involved in the motion and usually flow in the same direction as the cells of the motile strain.

These observations enabled us to conclude that colony movement is facilitated by the presence of a mucoid-watery cushion separating the colony from the agar surface, and that it depends to a great extent upon diffusion phenomena taking place between the sections of the medium with culture and those free of culture.

The displacement ability persisted in the Petri dishes even ten days after the inoculation. This confirms the ability of the bacterial mass to attract significant amounts of moisture from the nutrient medium, which ability, in turn, is directly dependent upon the formation of hydrophilic metabolites by the culture.

## SUMMARY

1. Bacillus coloniomobile shows displacement over the agar surface of round, bullet-like colonies, of small groups of cells, of thread-like forms and even of huge cell aggregates as large as 0.9 × 1.3 cm.

2. The movement of the cell formations may be translational, rotational or both.

3. Infrequently there are formations of tribolite-like figures of cell aggregates—these occur over destruction changes in the gel of the medium.

4. The migration rate of the bullet-like colonies may be as high as 256μ per 1 min. The rotation rate varies from $\omega = 0.019$ to 0.523 radian per sec, which is 20-200 times greater than that recorded by other authors. Movement along the ring of the thread-like forms proceeds at a similar rate.

5. Mobility of the colonies is influenced by the agar and sodium chloride content of the medium, as well as by the thickness of the nutrient layer, i.e., by the abundance of nutrients and the accumulation of metabolites.

6. It proved impossible to demonstrate that synchronous movement of flagella is the basis of migration of mobile colonies. However, it was noted that of great significance is fluidity of the bacterial mass and the difference in diffusion forces between areas of the medium occupied by bacteria and free of them.

7. The following observations confirm that migration of the bacterial aggregates is a function of the concentration of metabolites and of fresh nutrients: a) parallel and fan-like movement of the colony; b) colony movement leaving a trace in the form of a question mark; c) localization of the pathways of the migrating colonies with respect to the general lawn; d) formation of ring-like forms from long thread-like ones and of the sausage-like stretched bacterial mass and their circulatory movement; e) diffuse dispersion of the cells at the beginning of development of the culture; f) involvement in the diffusion dispersion over the surface of the medium of heterogenous cells belonging to immobile bacterial species, etc.

8. It is for the first time that the following phenomena have been recorded: a) extremely high rate of rotation; b) presence of noncellular traces after migration of round bullet-like colonies, of cell aggregates and of thread-like forms; c) formation of trilobite-like figures; d) formation of ring-like figures from sausage-like stretched cellular masses; e) migration of cell aggregates; e) significance of diffusion phenomena for migration of cellular elements.

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# SPECIES CHARACTERISTICS AND ANTIBIOTIC PROPERTIES OF ACTINOMYCETES ISOLATED FROM PRIMORIE SOIL

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In recent years great attention has been devoted to a study of the species content of antibiotic-producing Actinomyces in the soil of the USSR. These investigations have been carried on from the far north to the southern portion of the country (Aleshina and Makarovskaya, 1948; Egorov and Polin, 1955; Korenyako and Kuchaeva, 1955; Zharikova et al., 1958; Kuznetsov, 1958, 1959). The least studied in this regard has been the soil of the Far East (Zharikova et al., 1958). The soils of Primorie have not been studied at all.

The majority of investigators, in studying the ecology of antibiotic-producing Actinomyces, have devoted their attention to an elucidation of the role of the soil types, their fertility and covering flora. The distribution of antibiotic-producing Actinomyces in the soil has not been studied along genetic lines.

The aim of the present work is the study of the species content and antibiotic properties of Actinomyces which are encountered in different types of soil in Primorie, and to elucidate the peculiar distribution of the cultures in the soil according to genetic lines.

## METHODS

The method of isolating and culturing Actinomyces was reported in a previous work (Tumarkin, 1958). The determination of species of Actinomyces was carried out principally by the criteria of Krasil'nikov (1949) and Gauze (1957).

Preparatory selection of all isolated Actinomyces according to their antibiotic properties was carried out with three test cultures: Staphylococcus aureus, Salmonella typhosa (Bacterium typhosum) and Shigella dysenteriae (Bacterium dysenteriae). The Actinomyces whose antibiotic properties were established in the test cultures were further studied according to their action on a broader bacterial spectrum.

The soil samples were taken from seven soil cuts. Short characterization of the soil samples is presented in Table 1.

## EXPERIMENTAL RESULTS

In Table 2 the species characteristics of the antibiotic-producing Actinomyces isolated from the soils of Primorie are presented. The data show that in the soils of Primorie the species content of antibiotic-producing Actinomyces is rather variable. In the table 28 species are enumerated for which the species characteristics were established, but in fact the number of isolated species was greater so that the species identification was not established in a number of the Actinomyces (12 strains). The greatest species variation in antibiotic-producing Actinomyces, as is seen in Table 2, occurred in the cultivated soddy-podzol soil. Most frequently encountered in the soils were: Actinomyces chromogenes, Actinomyces cylindrosporus, Actinomyces globisporus and Actinomyces griseus. Attention is directed to the absence in the brown forest soil and in the brown highland soil of the widely distributed species such as A. griseus. Indeed, this species is widely distributed in the soddy-podzol region, especially in the cultivated soil. In brown forest and brown highland soils, A. chromogenes and A. cylindrosporus predominated. The results of the study of the distribution of the various forms of antibiotic-producing Actinomyces accord-

TABLE 1
Characteristics of the Soil Samples

| Place from which soil sample was taken | Soils |
|---|---|
| Maritime experimental station | Soddy-podzol cultivated (usual plowing) |
| The same | Soddy-podzol cultivated (deep plowing) |
| Cross-section No. 26, the same | Soddy-podzol (a bed lying for twenty-five years fallow, a small area in the middle of an arable field) |
| Cross-section No. 1, botanical gardens (19 km from Vladivostok) | Brown forest soil |
| Cross-section No. 25, Suputinsky Forest Preserve | Brown wooded highland soil (the cross-section was situated at 500 m above a plantation) |
| Cross-section No. 21, floodland of the river Mo (taken 6 km from Kharol') | Salty marshland |
| Cross-section No. D-40, floodland of the river Ussuri | Soddy-podzol |

The soil samples in the described cross-section were received from A. V. Mizerova, of the Primorie subsidiary station of the AN SSSR, to whom the authors wish to express their gratitude.

TABLE 2
Distribution of Various Forms of Antibiotic-Producing Actinomyces in the Soil of Primorie

| Species of Actinomyces | Actinomyces isolated total | | Soils | | | | | |
|---|---|---|---|---|---|---|---|---|
| | strain number | % of total | soddy-podzol cultivated (usual plowing) | soddy-podzol cultivated (deep plowing) | cross-section no. 26, soddy-podzol | cross-section no. 25, brown highland soil | cross-section no. 21, salty marshland | cross-section no. 1, brown forest soil |
| A. griseus Krainsky | 35 | 26 | 11 | 9 | 10 | 0 | 5 | 0 |
| A. griseovariabilis **Krasil'nikov** | 6 | 4.5 | 2 | 1 | 2 | 0 | 1 | 0 |
| A. iverini **Gauze** *et al.* | 1 | 0.7 | 0 | 1 | 0 | 0 | 0 | 0 |
| A. griseus zonatus **subsp. n. Krasil'nikov** | 1 | 0.7 | 0 | 1 | 0 | 0 | 0 | 0 |
| A. globisrorus **Krasil'nikov** | 18 | 14 | 3 | 11 | 1 | 2 | 1 | 0 |
| A. globisporus circulatus **subsp. n. Krasil'nikov** | 2 | 1.5 | 0 | 1 | 0 | 0 | 0 | 1 |
| A. phaeochromogenes Conn | 10 | 7 | 3 | 0 | 2 | 5 | 0 | 0 |
| A. chromogenes **Krasil'nikov** | 16 | 12 | 7 | 3 | 1 | 1 | 1 | 3 |
| A. cylindrosporus **Krasil'nikov** | 12 | 9 | 3 | 6 | 0 | 3 | 0 | 0 |
| A. fumosus **Krasil'nikov** | 5 | 3.5 | 1 | 2 | 1 | 0 | 1 | 0 |
| A. chromofuscus **Gauze** *et al.* | 1 | 0.7 | 0 | 1 | 0 | 0 | 0 | 0 |
| A. nigrescens **Gauze** *et al.* | 4 | 3 | 1 | 1 | 2 | 0 | 0 | 0 |
| A. griseolus **Krasil'nikov** | 2 | 1.5 | 0 | 0 | 0 | 1 | 0 | 1 |
| A. fumanus **Gauze** *et al.* | 3 | 2.2 | 0 | 2 | 0 | 0 | 1 | 0 |
| A. umbrinus **Gauze** *et al.* | 1 | 0.7 | 0 | 1 | 0 | 0 | 0 | 0 |
| A. longispororuber **Krasil'nikov** | 1 | 0.7 | 0 | 1 | 0 | 0 | 0 | 0 |
| A. longisporus **Krasil'nikov** | 2 | 1.5 | 0 | 0 | 0 | 0 | 2 | 0 |
| A. albidoflavus Duche | 2 | 1.5 | 2 | 0 | 0 | 0 | 0 | 0 |
| A. flavus. Krainsky emmend. **Krasil'nikov** | 1 | 0.7 | 1 | 0 | 0 | 0 | 0 | 0 |
| A. flavidoviridis **Krasil'nikov** | 3 | 2.2 | 0 | 0 | 1 | 0 | 2 | 0 |
| A. flaveolus var rectus **Gauze** *et al.* | 1 | 0.7 | 0 | 0 | 0 | 0 | 1 | 0 |
| A. flavidovirens **Gauze** *et al.* | 2 | 1.5 | 1 | 0 | 1 | 0 | 0 | 0 |
| A. longisporoflavus **Krasil'nikov** | 1 | 0.7 | 0 | 0 | 1 | 0 | 0 | 0 |
| A. rubiginosus **Gauze** *et. al.* | 1 | 0.7 | 0 | 0 | 1 | 0 | 0 | 0 |
| A. fradiae Waksman et Curtis | 1 | 0.7 | 1 | 0 | 0 | 0 | 0 | 0 |
| A. violaceus Gasperini | 1 | 0.7 | 0 | 0 | 0 | 0 | 1 | 0 |
| A. lavendulae Waksman et Curtis | 1 | 0.7 | 0 | 1 | 0 | 0 | 0 | 0 |
| A. violaceochromogenes **Krasil'nikov** | 1 | 0.7 | 0 | 1 | 0 | 0 | 0 | 0 |

TABLE 3

Distribution of Various Species of Antibiotic-Producing Actinomyces according to Genetic Line (cultivated soils)

| Soil | Genetic lines | A. griseus | A. griseovariabilis | A. iverini | A. griseus zonatus n. subsp. | A. globisporus | A. globisporus circulat. n. subsp. | A. phaeochromogenes | A. flavidovirens | A. cylindrosporus | A. chromogenes | A. violaceochromogenes | A. fumosus | A. chromofuscus | A. nigrescens | A. fumanus | A. umbrinus | A. longispororuber | A. albidoflavus | A. flavus | A. fradiae | A. lavendulae |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Soddy-podzol cultivated (usual plowing) | $A_1$ 0—10 | 4 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $A_1^1$ 10—20 | 2 | 0 | 0 | 0 | 1 | 0 | 2 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $A_2$ 25—35 | 2 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 4 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | $B_2$ 60—70 | 3 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 |
| Soddy-podzol cultivated (deep plowing) | $A_1$ 0—10 | 1 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | $A_1^1$ 10—20 | 2 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 4 | 3 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| | $A_2$ 25—35 | 5 | 1 | 0 | 0 | 3 | 1 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $B_2$ 60—70 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Summarized data of the two soils | $A_1$ 0—10 | 5 | 0 | 1 | 0 | 4 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | $A_1^1$ 10—20 | 4 | 0 | 0 | 0 | 6 | 0 | 2 | 0 | 5 | 5 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 |
| | $A_2$ 25—35 | 7 | 2 | 0 | 0 | 3 | 1 | 1 | 0 | 3 | 4 | 0 | 2 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 |
| | $B_2$ 60—70 | 4 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 2 | 1 | 0 | 0 |

TABLE 4

Distribution of Different Species of Antibiotic-Producing Actinomyces according to Genetic Line (virgin soils)

| Soil | Genetic line | A. griseus | A. griseovariabilis | A. rubiginosus | A. globisporus | A. globisporus circulatus | A. phaeochromogenes | A. flavidovirens | A. chromogenes | A. cylindrosporus | A. fumosus | A. nigrescens | A. fumanus | A. griseolus | A. longisporus | A. longosporoflavus | A. flaveolus var. rectus | A. flavoviridis | A. violaceus |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Soddy-podzol (cross-section 26) | $A_1$ 0—7 | | | | | | | | | | | | | | | | | | |
| | $A_1^1$ 7—14 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $A_2$ 17—30 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $B_1^1$ 30—43 | 6 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | $B_2$ 43—50 | 2 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| | | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Brown forest soil (cross-section 1) | $A_1$ 3—10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $A_2$ 13—27 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | BC 21—45 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Brown highland soil (cross-section 25) | $A_1$ 2—12 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | $A_1^1$ 12—28 | 0 | 0 | 0 | 2 | 0 | 5 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | BC 28—42 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Salty marshland (cross-section 21) | $A_1$ 0—7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | $A_1^1$ 7—17 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| | $A_2$ 17—30 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| | $B_1$ 30—43 | 2 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| | B 43—50 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Summarized data | $A_1$ | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | $A_1^1$ | 0 | 0 | 0 | 3 | 0 | 5 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| | $A_2$ | 8 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 3 | 0 |
| | $B_1$ | 4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 |
| | $B_2$ | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

TABLE 5

Distribution of Actinomyces Producing Antibiotics against S. aureus, S. dysenteriae and S. typhosa in the Soils of Primore

| Cross-section No. | Soils | Soil samples investigated | Actinomyces isolated, total | No. antagonistic to one of the three test cultures | % |
|---|---|---|---|---|---|
| D-40 | Soddy-podzol | 3 | 45 | 7 | 15 |
| R-26 | The same | 5 | 135 | 42 | 31 |
| R-25 | Brown highland soil | 4 | 58 | 13 | 22 |
| | Soddy-podzol cultivated (usual plowing) | 4 | 343 | 57 | 17 |
| | Soddy-podzol cultivated (deep plowing) | 4 | 387 | 65 | 17 |
| R-21 | Salty marshland | 5 | 76 | 21 | 30 |
| R-1 | Brown forest soil | 3 | 45 | 7 | 15 |

TABLE 6

Antagonistic Properties of Actinomyces Isolated from the Soils of Primorie

| Strains of Actinomyces investigated | Suppressed the growth of | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Staph. aureus | | Mycobacterium B. | | Bact. pyocyaneum | | Bact. prodigiosum | | Bact. proteus ammonium | | Bact. coli | | Bact. dysenteriae | | Bact. paratyphi B | |
| | no. | % | no. | % | no. | % | no. | % | no. | % | no. | % | no. | % | no. | % |
| 130 | 130 | 100 | 55 | 41 | 5 | 4 | 5 | 4 | 8 | 6 | 15 | 11 | 15 | 11 | 11 | 8 |

ing to genetic lines in two cultivated soils and four virgin soils are given in Tables 3 and 4. From an analysis of the data in Table 3 it is seen that the genetics of lines $A_1$, $A'_1$, and $A_2$ predominantly contained A. chromogenes, A. cylindrosporus, A. fumosus, A. pharochromogenes, which were almost never encountered in line B. From line B of these soils Actinomyces flavus, Actinomyces nigrescens, Actinomyces griseus var. zonatus and Actinomyces chromofuscus were isolated. Such species as A. griseus and Actinomyces griseovariabilis are encountered in all the soils. The antibiotic-producing species Actinomyces fumanus, Actinomyces umbrinus were observed in the genetic lines $A_1$ and $A_2$, but were not observed in line $B_2$.

The species distribution of the antibiotic-producing Actinomyces in virgin soils as seen in Table 4 follows the same general rule as cultivated soils. Thus, A. chromogenes, A. cylindrosporus, A. fumanus are observed in the upper soil lines. For antibiotic-producing Actinomyces of the species Actinomyces longisporoflavus, Actinomyces flaveolus var. rectus they are characteristically situated in the deeper soil lines—$B_1$ and $B_2$ and for Actinomyces flavoviridis in line $A_2$. Actinomyces albidoflavus in cultivated soddy-podzol and virgin soddy-podzol were observed in lines $B_2$ and $B_1$. Thus it can be concluded that several species of antibiotic-producing Actinomyces are preferentially encountered in predetermined genetic lines.

In Table 5 the characteristics of the quantitative content in different soils of Actinomyces is given and it shows that antibiotic properties act on at least one of the three test cultures (S. aureus, S. dysenteriae and S. typhosa). As is seen in Table 5, the percentage of active cultures of Actinomyces varies between 15 and 30. It should be noted that Actinomyces which suppressed the growth of typhoid and dysentary bacilli in all experiments

also suppressed the growth of S. aureus. The Actinomyces which had antagonistic properties to the aforementioned test cultures were studied with a broader bacterial spectrum.

In Table 6 the summarized data are given on the antibacterial properties of 130 strains of antibiotic-producing Actinomyces isolated from the soil of Primorie belonging to 31 different species [of them 28 species are shown in Table 2 and three species were isolated from soil samples obtained from cross section D-40 (see Table 1)].

## SUMMARY

1. The Primorie soils are characterized by a wide species composition of the actinomycete antagonists. Most frequently there occur in these soils the following species: Actinomyces griseus, Actinomyces cylindrosporus, Actinomyces chromogenes and Actinomyces globisporus.

2. In brown forest soils no cultures of Actinomyces griseus species were found.

3. Preferential distribution of some actinomycete species along definite soil horizons has been noted: Actinomyces chromogenes, Actinomyces cylindrosporus and Actinomyces phaechromogenes are distributed mostly in turf-podzol (both virgin and cultivated) and in brown forest soils of the $A_1$ and $A_2$ genetic horizons, while Actinomyces flavus, Actinomyces albidoflavus, Actinomyces flaveolus var. rectus and Actinomyces flavidovirens occur in the B horizon.

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**All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.**

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# SUCRASE ACTIVITY IN THE SOIL

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All processes of conversion of organic matter in the soil are inseparably connected with enzyme activity.

That the main potentiality of fermentative reactions in the soil is outside of the cells of living organisms is not to be doubted, since both the number of enzymes with preservation of their activity and their isolation after the disappearance of some or other microorganisms have been demonstrated (Hofman and Hofman, 1955; Hofman, 1956). The isolation of enzymes from the roots of higher plants belonging to different families has also been reported (Krasil'nikov, 1952; Kuprevich, 1949, 1954). Consequently, fermentative activity is a summated activity —involving microorganisms and the roots of higher plants.

From investigations devoted to soil enzymes it is seen that the most varied groups of enzymes are found in the soil. The activity of these may comprise the rapidity of decomposition of various organic compounds (Galstyan, 1957; Drobnik, 1957; Kuprevich, 1951; Hofman and Braumlich, 1955; Hofman and Scegerer, 1951).

One of the most widespread enzymes in microorganisms as well as in plants is sucrase, which hydrolyses sucrose—one of the intermediate products in the decomposition of various carbohydrates which form part of the plant residues.

We investigated the dynamics of sucrase activity in turf-podzol heavy loams in the Leningrad Agricultural Institute experimental fields under different cultivation and different agronomic backgrounds.

Some of the plots had lain fallow for 18 years with a prevalent cereal grass vegetation; the rest were two parcels of cultivated land. In one of them the customary agrotechnic (part field with the usual agricultural background) was used, but on the other, in 1956, with a view to accelerated cultivation of the turf-podzol loams the soil was plowed under to 38 cm with an admixture of the humus horizon and the alluvial part with 80 T/hectare peat-manure (1:1) and 8 T/hectare slaked lime (the parcel of land with high agrofon). In 1958 both plots were sown with "Plentiful" winter wheat. In addition, in 1959 the winter wheat field was sown with an oat-peat mixture on the first plot and grass from the first year of use (clover and timothy) on the second.

For comparison, an area of dead coniferous forest cover was laid on the turf-podzol heavy loamy soil at Semrino station (Leningrad region). During the vegetation season 1958-1959 on this area samples of the model soil were taken and the sucrase activity determined iodometrically according to the method of Hofmann and Scegerer (1951) in triplicate tests.

The sucrase activity at depths of 0-10 cm under various types of cultivation during the vegetation season 1958-1959 is shown in Fig. 1. In the horizons from 20-30 and 30-40 cm the sucrase activity was quite insignificant (0.8-0.2 ml 0.1 N $Na_2S_2O_3$ in 1 g of soil) with the exception of fields with high agricultural background, in which the sucrase activity in the 20-30 cm horizon reached 4 ml 0.1 N $Na_2S_2O_3$ in specimens taken at separate times.

Katsnel'son and Ershov (1958), studying the sucrase activity in soils from the Karel'sk ASSR, also found that in all mineral soils the greatest activity was in the highest horizons, after which the activity fell sharply. Jagnow

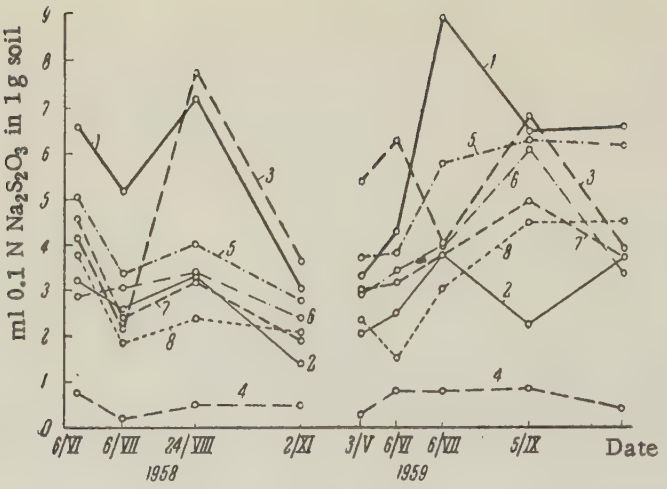


Fig. 1. Sucrase activity in soils under different types of cultivation during the vegetation period 1958-1959. Horizons:
1) 1-10; 2) 10-20 (fallow); 3) 5-13; 4) 13-26 (wooded);
5) 0-10; 6) 10-20 (field with high agricultural background);
7) 0-10; 8) 10-20 (field with ordinary agricultural background).

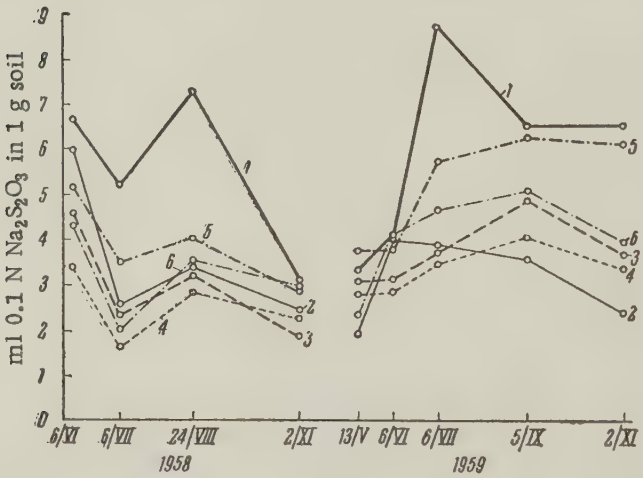


Fig. 2. Sucrase activity in soils containing plants and without plants (0-10 cm horizon). Fallow land: 1) with plants; 2) without plants. Fields with ordinary agricultural background: 3) with plants; 4) without plants. Fields with high agricultural background: 5) with plants; 6) without plants.

(1958) and Galstyan (1957) also noted that the inverting capacity of soils decreases with the depth according to the soil profile.

Sucrase activity in considerable degree depends on the character of the land. The greatest sucrase activity is observed in fallow lands in the 0-10 cm horizon. It is somewhat lower in wooded soils and even lower in tilled soils, being higher in tilled soils with high agricultural backgrounds than in tilled soils with the usual agricultural background.

Kuprevich considers that the enzymatic activity in fallow and virgin soils is somewhat greater than in soils occupied for a long time by crop culture, since the surface layers of virgin soil are penetrated to a greater degree by the root system of grassy vegetation and contain considerably more organic matter per unit volume (Kuprevich,

1958). Kuprevich's postulate is fully confirmed by our experimental data on sucrase activity. Katsnel'son and Ershov (1958) also have established that in certain instances the sucrase activity in noncultivated fields is greater than in cultivated ones.

The sucrase activity changes over the course of the year. In 1958 we observed two maxima in sucrose activity, one in the spring and the second at the end of summer. We encountered a similar picture in 1959 also in wooded soils.

In all remaining experimental variants in 1959 we observed a few which had different dynamics. In fallow soils the greatest sucrase activity was in mid-summer. In cultivated land it reached maximal value at the beginning of September.

Seasonal sucrase activity has been observed by a number of authors (Galstyan, 1957; Katsnel'son and Ershov, 1958; Ambroz, 1956), Ambroz (1956) and Galstyan (1959) reporting that the greatest enzyme activity is observed in the summer period.

We determined sucrase activity not only in plant-containing fields but also in unplanted fields. The plant cover on these fields was systematically removed during two vegetation periods.

Comparing the sucrase activity in plots with plants and without, it is evident that the activity is always greater in planted plots (Fig. 2). We propose that the increase in sucrase activity in planted areas is connected with a higher number of microorganisms in the plant rhizosphere, the secretion of enzymes by the plant roots and the enrichment of the soil by the plant residues.

In reality, the investigations showed that extremely great numbers of microorganisms are concentrated around the roots of plants. This occurs because plants secrete from their roots into the soil a quantity of organic compounds (sugars and organic acids).

In addition, plants continuously throw off from root surfaces dead epidermal cells and root hairs which also serve as a source of nourishment for the microorganisms.

The studies of Krasil'nikov (1952) and Kuprevich (1949, 1954) have proved that root systems, and especially their fine endings, secrete various enzymes into the soil throughout their lives and, in dying, give their entire enzymatic apparatus into the soil.

We have also studied the sucrase activity in a number of soils from the European part of the USSR.

The following soils were taken for the investigation: 1) turf-medium podzol loamy soil in clay bands under grass of three years' use, Leningrad region; 2) sandy, humus-alluvial podzol in ferruginous, medium granular sand, mixed forest, Karel' isthmus; 3) turf-heavy podzol loamy soil on loam cover, fir forest, Leningrad region; 4) turf-weak podzol loamy soil in carbonaceous moraine, put into common pasture, Leningrad region; 5) gray wood podzol loamy soil on wood loam, young birch forest, Orlov region; 6) typical strong chernozem, loamy soil in loess-like loam, virgin soil, Kursk region, Central Chernozem State Preserve; 7) chestnut salinaceous loamy soil in pale yellow loess, irrigated fields, Cherson region. All assays were carried out on soil taken in the year of the investigation (1957).

Sucrase activity in the profile of the soils studied is shown in Fig. 3. *

From the data presented it is clear that the greatest sucrase activity within the limits of the entire 50 cm thickness is possessed by the strong virgin land chernozem. The enzyme activity gradually declines with the depth in the profile of gray loess and chestnut soils. In the group of turf-podzol soils there stands out the soil in carbonaceous moraines, which is characterized by a high activity within the boundaries of the humus horizon.

In all the remaining soils of this group the sucrase activity is not high, even in the top part of the humus horizon, and it very sharply declines with depth, clearly correlating with the distribution of humus in the profile.

Thus, changes in sucrase activity along the profile correlate quite well with the humus distribution. This interdependence may be understood if one takes into account that the distribution of humus along the profile is conditioned by the basic depth of penetration of plant root systems, decay of which is accompanied by soil microorganisms.

* Humus assay by the method of Tyurin.

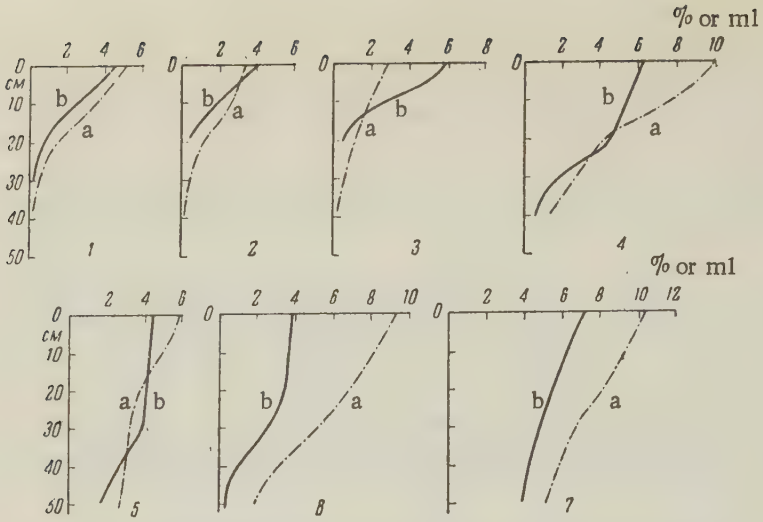


Fig. 3. Sucrase activity and humus content in soils. a) Sucrase activity in milliliters 0.1 N $Na_2S_2O_3$ in 1 g soil; b) humus content in percent. 1) Turf-medium podzol loamy soil in clay bands under grass of three years' use, Leningrad region; 2) sandy, humus-alluvial podzol in ferruginous, medium granular sand, mixed forest, Karel' isthmus; 3) turf-heavy podzol loamy soil on loam cover, fir forest, Leningrad region, 4) turf-weak podzol loamy soil in carbonaceous moraine, put into common pasture, Leningrad region; 5) gray wood podzol loamy soil on wood loam, young birch forest, Orlov region; 6) typical strong chernozem, loamy soil in loess-like loam, virgin soil, Kursk region, Central Chernozem State Preserve; 7) chestnut salinaceous loamy soil in pale yellow loess, irrigated fields, Cherson region. All assays were carried out on soil taken in the year of the investigation (1957).

1. Sucrase activity in turf-podzol heavy loamy soil in various cultivated lands changes during the vegetation season.

2. Sucrase activity is considerably higher in plant-containing soils than in soils without plants.

3. In the major basic soil types in the European part of the USSR the sucrase activity appreciably decreases with the depth, correlating with the distribution of humus along the soil profile.

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# DISTRIBUTION OF FILAMENTOUS CLUSTER MICROORGANISMS (Krasil'nikoviae) IN THE DEPTHS OF SEAS AND OCEANS

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Translated from Mikrobiologiya, Vol. 30, No. 1, pp. 110-112,
January-February, 1961
Original article submitted April 5, 1960

We have previously established (Kriss and Mitskevich, 1957) that the distinctive filamentous cluster microorganisms that inhabit the waters of seas and oceans may be observed in mature form and considerable numbers on glass plates after 6 hr following the submersion of these plates at various depths. Considering that the organisms were not found on filter membranes through which were passed 20–30–50 ml of water taken from those levels where filamentous cluster microorganisms had been found on the glass, we proposed that the development of the organisms had proceeded on the glass and was not merely the settling out of previously formed filaments bearing head structures.

However, so great a rapidity of development seemed startling and therefore the investigation was continued. In the open reaches of the Black Sea glass plates were lowered to the depths of 25, 100, 200, 300, 500, 750 and 1000 m and were kept there for various periods ranging from 30 min to 2 hr 45 min. As Table 1 shows, even on short exposure (30 min to one hour) thick interlacings of filamentous cluster microorganisms were found.

TABLE 1

Rapidity of Appearance of Filamentous Cluster Microorganisms on Glass Plates after Submersion of the Latter into the Black Sea

| Depth plates were submerged, m | December, 1957 | | | | August, 1958 | February, 1959 | |
|---|---|---|---|---|---|---|---|
| | Length of exposure of the plates in the water | | | | | | |
| | 2 hr, 45 min* | 2 hr* | 1 hr* | 30 min* | 15 min | 5 min | 5 min |
| 25 | Single individuals | Single individuals | Single individuals | Single individuals | | | |
| 100 | Interlaced networks | Interlaced networks | Interlaced networks | Interlaced networks | Interlaced networks | Interlaced networks | Interlaced networks |
| 200 | The same | The same | The same | The same | | | |
| 300 | The same | The same | The same | The same | | | |
| 500 | Single individuals | | The same | The same | | | |
| 750 | Interlaced networks | Interlaced networks | The same | The same | | | |
| 1000 | The same | The same | The same | The same | | | |

*Indicated exposure is for the least deeply submerged plate; for the remaining ones exposure time is much greater since in the simultaneous lowering of plates to the appropriate levels the difference in the time underwater between the topmost and bottommost (1000 m) plate is about one hour.

TABLE 2

Distribution of Filamentous Cluster Microorganisms in the Central Part of the Pacific and Atlantic Oceans

| Depth plates were submerged, m | Pacific Ocean, 1958 | | | | Atlantic Ocean, 1959 | | |
|---|---|---|---|---|---|---|---|
| | 3777 | 3782 | 3812 | 3823 | 347 | 363 | 377 |
| | 32°48′3″ N 174°02′ W | 24°03′2″ N 173°31′ W | 14°49′ S 172°56′ W | 23°26′5″ S 174°39′54″ W | 66°00′ N 30°00′ W | 33°30′ N 30°00′ W | 00°05′ N 30°00′ W |
| | Length of exposure of plates, hr | | | | | | |
| | 6 | 6 | 12 | 6 | 10 | 40 | 40 |
| 5 | 20 | 2 | 500 | | 0 | | |
| 10 | 2 | 30 | 1900 | 0 | | | |
| 15 | 800 | | | | | | |
| 25 | 50 | 0 | 2100 | 0 | 4160 | | |
| 35 | 0 | | | | | | |
| 50 | 310 | 210 | 240 | 20 | 13150 | 840 | 5050 |
| 75 | 200 | 25 | 1100 | 2000 | 70 | | |
| 100 | | 65 | 390 | 0 | | 990 | 13 |
| 150 | | 145 | 1300 | 0 | | | |
| 200 | | 130 | 0 | 4 | | 130 | 130 |
| 250 | | | 365 | | | | |
| 300 | | 60 | 20 | | | | |
| 400 | | | 80 | | | | |
| 500 | | | 0 | | | 90 | 45 |
| 1000 | | | | | | 680 | 105 |

Figures express total number (in a series of instances rounded off to tens) of filaments bearing head structures on a glass area of 15 $cm^2$.

Such short periods of time are obviously insufficient for the formation of filaments bearing hard structures on the plates and, after the next experiment, it became definitely evident that single individuals or networks and masses of filamentous cluster microorganisms are mechanically affixed to the glass plates which were submerged in the depths. Plates were submerged to 100 m and immediately drawn up. Although the descent and ascent of the plates took only 5 min, filaments bearing head structures were already observed on them, singly or as masses and interlacing networks.

At the same time it was ascertained that the bathometric samples of water from different depths in the Black Sea did not contain filamentous cluster microorganisms in volumes of 500 to 1000 ml. To be convinced of this, we filtered these volumes through ultrafiltration membranes and then examined them under the microscope.

These data suggest to us that the filamentous cluster microorganisms are usually distributed in the waters of seas and oceans, not in a diffuse fashion, but instead form layers and accumulations at definite depths. It suffices merely to pass glass objectives through such a layer or accumulation in order to catch a considerable number of filamentous cluster microorganisms, usually interlaced as long rods, visible to the naked eye.

The rotation of the rope, especially in rapid lowering, has a detrimental effect on the "catch." We became convinced of this on lowering glass plates measuring 12×14 cm fixed to the rope in a horizontal position by means of rubber supports. It could be seen how the glass plates revolved this way and that on the descent and ascent; obviously, such motion in the water pushes away the masses of filamentous cluster microorganisms, judging by the relatively small quantity of microorganisms on the large plates in comparison with the glass objectives.

Evidently, not merely one layer of filamentous cluster microorganisms is formed in the depths of seas and oceans (Kriss and Mitskevich, 1957, Table 1) since frequently the quantity of these organisms in deeper-lying horizons is greater than in more superficial ones. These layers are presumably arranged at the junction of water masses of different densities. In this way we explain the comparatively greater numbers of filamentous cluster microorganisms at the higher and lower boundaries of the Atlantic currents in the region of the North Pole.

The wide distribution of the filamentous cluster microorganisms in the global ocean now has received new confirmation thanks to the studies which were carried out in the central part of the Pacific (expedition on the

"Champion" in 1958) and along the 30th meridian in the Atlantic (expedition on the "Lomonosov" in 1959).

We have established, by methods described earlier and in supplement to data already published (Kriss and Mitskevich, 1957), that the filamentous cluster microorganisms are found also at different depths in subtropical and tropical-equatorial regions (Table 2) and that, consequently, their geographical area in the Pacific Ocean stretches from polar latitudes to the equator.

## SUMMARY

1. The investigations carried out suggest that filamentous cluster microorganisms (Krasil'nikoviae) are distributed in the seas and oceans, not in a diffuse manner, but form accumulations at definite depths. These accumulations might presumably be arranged at the junction of water masses of different density.

2. The area of filamentous cluster microorganisms comprises all the geographical zones of the global ocean from polar latitudes to the equator.

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# SEASONAL CHANGES IN NUMBERS OF BACTERIOPLANKTON IN WATERS OF LAKE BAIKAL

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Translated from Mikrobiologiya Vol. 30, No.1, pp. 113-117, 1961
Original article submitted January 19, 1960

The study of the biological productivity of waters entails a determination of the role of bacteria in the cycle of substances. One of the ways of approaching a solution of this problem is a count of the total numbers of bacteria by direct microscopy (Razumov [1932]).

The first determinations of the total numbers of bacteria in Lake Baikal were carried out by Kuznetsov [1951] and later by Rodina [1954] in the open waters of the lake. In 1951-1952 periodic observations on the numbers of bacterioplankton in different seasons were organized at the Baikal Limnological Station of the Biological and Geographical Institute of Irkutsk University in Bol'shie Koty (Kozhova [1956]). In 1953-1954 Romanov [1958] studied the fluctuations in numbers and the vertical distribution of bacterioplankton in the region of Listvenichnoe.

In 1956-1958 we continued the determinations of the total numbers of bacteria in the region of B. Koty in order to establish the features of the seasonal fluctuations of the bacterioplankton pupulation in these years and to compare the course of development of the bacterio-, phyto- and zooplankton.

We collected material at regular intervals one to three times a month at the same points as in 1951-1952. The region of investigation was typical of the deep open water of Baikal, where the waters are characterized by low mineral content, high transparency (to 25 m or more), low summer temperatures (to 12-14 C) and a weak (in comparison with other lakes) development of plankton. At certain periods, particularly after strong northeast winds, this region is influenced by the Selenga Bank waters, which are distinguished by a slightly different chemical composition and a greater plankton content.

We took samples in sterile flasks by means of a water sampler from standard hydrological horizons and filtered these samples through No. 2 membrane ultrafilters immediately after collection. The filters were stained with 1% carbol erythrosin and the bacteria were counted under a magnification of 1000 ×. The biomass was determined by calculating the volume of the individual cells, their density being taken as unity. The volume of the large, rarely occurring cocci was 9.2 $\mu^3$, the medium-sized coccoid forms 1.4 $\mu^3$, the long rod-shaped forms 1.6 $\mu^3$, and the short rods 3 $\mu^3$. At the same time as the bacterioplankton samples were taken, M. M. Kozhov and N. L. Antipova conducted observations on the development of phyto- and zooplankton. The phytoplankton was sampled with a water sampler, and the zooplankton with a large Juday net; these samples were analyzed by conventional methods.

The observations which we carried out (Figs. 1, 2) showed that the greatest numbers of bacteria during the course of the year were recorded in the following months—in August and September in 1957, and in the second half of July and in August in 1958, when the summer temperature stratification was established in Baikal. In the ice-cover period (February – March) and after the thawing of the ice, during the spring homothermy (June – first half of July), there are many fewer bacteria than in summer. The numbers of bacteria can be a little higher under the ice than in the following period of homothermy, as in 1957, for instance. The bacterial biomass begins to increase in early spring, and by summer the average values for the surface of the most densely populated 50 meter layer are 100 to 800-900 mg/$m^3$. This seasonal periodicity in the development of bacteria is in full

agreement with earlier results. For instance, Romanova [1958] observed early spring and summer maxima of the bacterial population. In previous investigations in 1952 we distinguished three maxima in the development of bacteria – a small one under the ice, a peak summer one, and an early autumn one. However, an analysis of the hydrometeorological data showed in that year there were probably only two maxima of the bacterial population, since the early autumn maximum was a continuation of the summer one, and the fall in bacterial concentration in August was due to the heated surface waters (11-12 C) being driven off by the strong northwest winds, and to the ascent of cold (4 - 6 C) deep water containing less bacterioplankton.

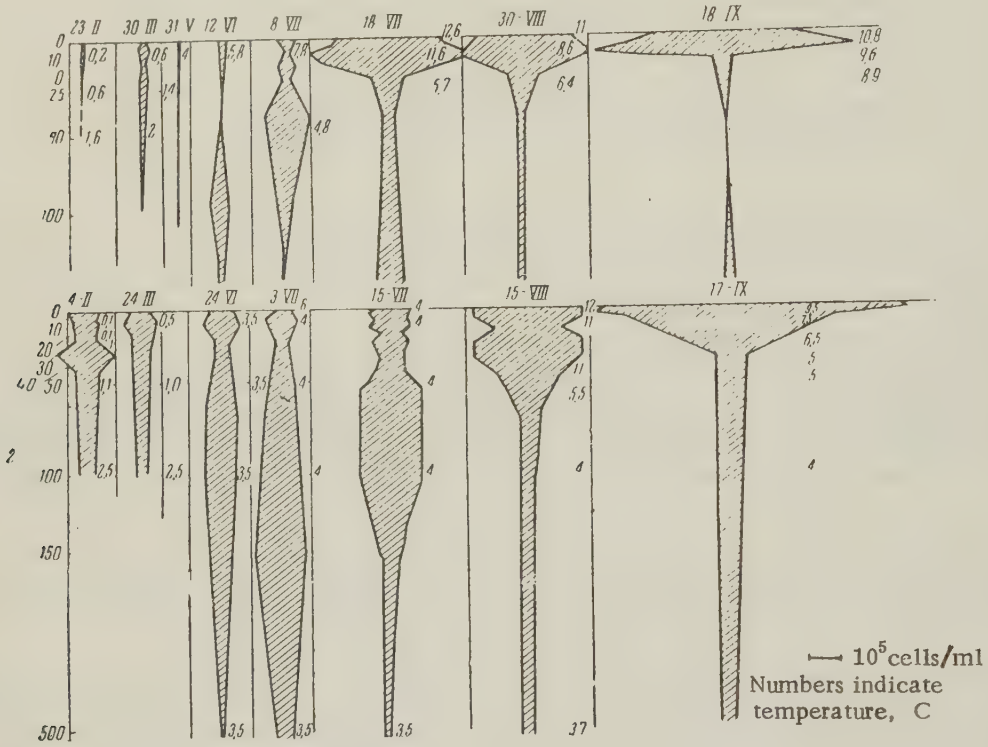


Fig. 1. Vertical distribution of bacterioplankton at different seasons in region of Koty Bay. 1) 1952; 2) 1957.

The vertical distribution of bacteria in different seasons also shows great variation. Comparing the results obtained in the region of Koty over several years (1952, 1956-1958) we can distinguish several types of vertical distribution, examples of which are given in Fig. 1. In the early spring ice-cover period (March-April) there are usually greater numbers of bacteria in the surface waters – from 0 to 5-10 m or to a depth of 20-30 m. After the ice thawed, in the period of homothermy, the bacteria were again unevenly distributed in the water: an increase of bacterial concentration in the 0-10 m layer can be noted and below a certain region of lower concentration there is another increase at depth 25-30 m or even 100 m. Only in certain cases were the bacteria uniformly distributed in this season. The most pronounced vertical stratification of bacteria begins with the summer heating of the waters and lasts throughout the entire summer period – from July (second half) to September. The greatest concentration of bacteria occurs in the 0 to 20-30 m layer, where there are 0.5-1 million cells per ml, but in the deeper layers they are much fewer in number – up to 100,000 cells per 1 ml. In autumn, in October, according to Kuznetsov's data [1957], the maximal numbers of bacteria occur at depth 25-50 m.

Thus, it is obvious that in Baikal the seasonal changes in bacterial population mainly affect the 0 to 50 m layer, and their greatest numbers are observed in summer. The numbers of bacteria may vary considerably in different years, but the order of magnitude remains the same. For instance, in August, 1948, the number of bacteria in 1 ml of the surface layer in the region of our investigations was 230,000 (Kuznetsov [1951]), in 1952 it was 800,000 (Kozhova [1956]), in 1954 it was 1 million (Romanova [1958]), and in 1957-1958, as we already mentioned, it reached 1 million.

The question arises – what is the cause of the seasonal fluctuations and the differences in the vertical distribution of bacteria in Lake Baikal? We know that the numbers of bacteria and their distribution depend on the presence of available nutrient substances. It is regarded as an established fact that in deep bodies of water the main source of food for bacteria is planktonic algae. This is confirmed by the existence of a direct relationship between the numbers of phytoplankton and bacteria, a feature noted by several authors (Guseva [1951]; Kriss [1959] and others) for waters of different types. Proceeding from this viewpoint, we (Kozhova [1956]), and also Romanova [1958], suggested that in Lake Baikal the seasonal course of development of the bacterioplankton was governed by the presence of nutrient substances in the form of developing (intravital excretions) and dying phytoplankton and, to some extent, zooplankton. And in fact, an increase in the numbers of bacteria was noted in the early spring and summer periods, when the growth of the phytoplankton was enhanced. As regards vertical distribution, the greatest concentrations of bacteria and algae were usually noted in the same layers (usually 0-50 m).

However, a careful comparison of the seasonal fluctuations of the bacterio- and phytoplankton in Lake Baikal revealed an interesting fact – the annual maxima of population and biomass of the bacterio- and phytoplankton occurred at different periods. The highest phytoplankton biomass in the open waters of Baikal, in contrast to other lakes, is usually found in the ice-cover period, when psychrophilic diatoms and peridinians develop. After the thawing of the ice, in the period of homothermy, the numbers of phytoplankton diminish. In summer another maximum is noted, but this maximum is never as high as that under the ice; the algal biomass is only tens of mg per $m^3$ in the trophogenic layer of water. The seasonal course of development of the phytoplankton in the period of our observations is shown in Fig. 2, and there we also show the biomass of bacteria in the most densely populated layer (0-50 m). The figure shows that the greatest maximum of the phytoplankton biomass in 1957 and 1958 occurred in early spring, under the ice, but that of the bacterioplankton occurred in summer. In spring the bacterial biomass was smaller than that of the algae, but in summer, on the other hand, it was many times greater than the latter: in summer the maximal phytoplankton biomass (according to Antipova's data) in 1956 was about 100 mg/$m^3$, in 1957 it was 20 mg/$m^3$, whereas the bacterial biomass in these years was 400-900 mg/$m^3$ on the average in the 0-50 m layer. We must mention that for the waters of central Baikal north of the Selenga Bank, where algae are usually more abundant, Kuznetsov [1951] determined the phytoplankton biomass as 0.4 g/$m^3$, and the bacterial biomass as 0.06-0.4 g/$m^3$. In the region of our investigations the algal biomass was at this time (according to Antipova's data) only a few tens of mg/$m^3$ and the bacterial biomass was 0.4 g/$m^3$. Hence, the ratio of the bacterial biomass to the algal biomass was approximately the same as in 1956-1958.

How are we to explain the fact that the highest annual maxima of development of bacteria and phytoplankton occur at different periods in the year? It would appear that in summer, when the algal biomass is low, production processes are much more intense than in spring and, in fact, much more organic matter is created than we can assess from its biomass. This is indicated, for instance, by results of determination of the energy of photosynthesis by the bottle method (Votintsev and Samarina [1957]), and also by the fact that at this time there is noted a minimal content of biogenic elements, which are consumed by the developing phytoplankton (Votintsev [1952]). Besides phytoplankton, the food of bacteria may also consist of dying zooplankton, which at this time develop in maximum numbers (Fig. 2).

Beside the phyto- and zooplankton we should mention another possible source of nutrient substances for bacteria – dissolved organic matter. According to Votintsev's data [1955], the bichromate oxygen absorption in summar in open Lake Baikal reaches 4 mg $O_2$ per liter. In addition to this, bacteria probably utilize organic substances excreted by algae. The existence of such excretions was established by Goryunova [1950, 1954] for diatoms and blue-green algae. Our assumption is based on the fact that in Baikal in July-August the water acquires a brownish-green color, and when it is filtered through a membrane filter it leaves a greenish deposit, which probably consists of organic matter excreted by algae.

Another favorable factor for the rapid development of bacteria in the summer period is the increased temperature of the water. As Figs. 1 and 2 show, the development of bacteria is intensified when the summer temperature stratification sets in, and their greatest numbers are associated with the heated surface layer 0-20-30 m. Below the thermocline the numbers of bacteria are much lower. The same feature was also noted by Romanova [1958].

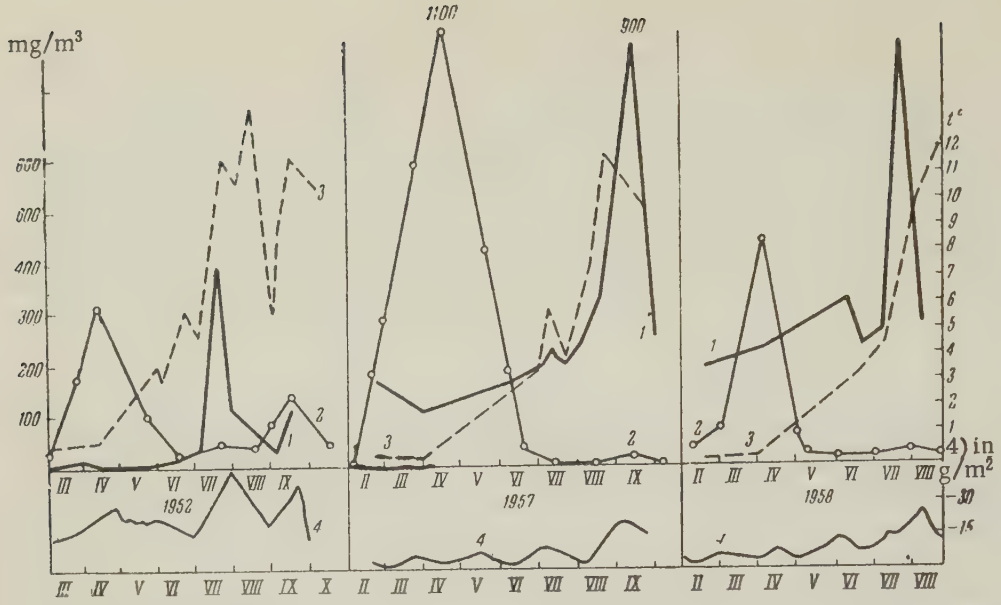


Fig. 2. Seasonal changes in biomass of bacterio-, phyto- and zooplankton and water temperature in region of Koty Bay in 1952, 1957 and 1958. 1) Bacterial biomass in mg/m³ in 0-50 m layer; 2) phytoplankton biomass in mg/m³ in 0-50 m layer; 3) temperature at surface; 4) zooplankton biomass in g under 1 m² in 0-250 m layer.

## SUMMARY

1. Observations over several years have shown that the greatest maximum in the numbers of bacterioplankton is the summer one (second half of July - September), when up to 1 million bacteria per ml are found, but the spring maximum is very weak.

2. In early spring and summer the maximal numbers of bacteria are found in the upper water layers, from 0 to 20-30 m. During the spring homothermy a maximum is noted in the upper layers or at depth 25-100 m, though in some cases the bacteria are dispersed more or less uniformly in the water.

3. The mass development of bacteria occurs in the period when the water is warmest and the zooplankton development reaches its maximum. The annual maxima of the numbers and biomass of phyto- and bacterioplankton occur at different times – the phytoplankton in spring, and the bacteria in summer.

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# DISTRIBUTION OF BACTERIAL FLORA IN AIR ABOVE CENTERS OF POPULATION AND REMOTE FROM THEM

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The biocomponent of the atmosphere of populated localities is closely related to the sanitational and topographical features of the underlying surface. The aerosol state of microorganisms ensures the possibility of their transfer over considerable distances according to the speed and direction of air currents. For instance, in March 1901, as Skrżyńska [1949] reports, dust clouds from south Algeria were blown over Denmark, England, and Russia. In 1928, bacteria-loaded dust clouds formed, as the author believes, in Central Asia were observed at a height of 600 m over Lvov and Ternopol. According to Soule's observations [1934], a culture of the hay bacillus was carried over a distance of several tens of kilometers during a dust storm. The survival rate of bacteria in air at great altitude is high. According to Stevens [1936], viable forms of Fungi Imperfecti have been found even at altitude 72, 395 feet (22.108 m).

Some questions concerning the microflora of the air above 250 m from the ground have still not been adequately studied. In particular, for instance, the question of the role of populated centers in the formation of the bacterial aerosols of the atmosphere cannot be regarded as settled. In our observations of 1957-1958 we determined the degree of bacterial contamination of the air in a vertical section in the latitude of Lvov and we studied the species composition of the aeromicroflora. The purpose of the 1959 investigations, in addition to the development of a new technique (Kishko and Filoppov [1959]), was to study the bacterial content of the upper air at various distances from Lvov, and also to investigate the zonal distribution of microorganisms in the air in the district of Nikolaev (35 km from Lvov).

For this purpose 242 air samples were collected during 1959. The air samples were taken by our suggested collector device (Kishko [1959 a, b]) in an LI-2 airplane.

## RESULTS OF INVESTIGATIONS

Table 1 gives the data on the aerial microflora at height 250 m over Lvov and at different distances from it.

TABLE 1

Air Microflora at 250 m Height on Passage of Airplane over Route Lvov-Nikolaev (April 7, 1959)

| Point of investigation | Microorganisms in 1 $m^3$ of air | Composition of microorganisms, % | | | |
|---|---|---|---|---|---|
| | | pigmented | sporing | colorless | fungi |
| Over Lvov | 1176 | 47.6 | 31.6 | 6.1 | 14.7 |
| 10 km from Lvov (over fields) | 628 | 21.9 | 51.6 | 8.6 | 17.9 |
| 15 km from Lvov (over forest) | 662 | 17.6 | 30.0 | 2.4 | 50.0 |
| Over Bobrka (25 km from Lvov) | 434 | 47.4 | 24.9 | 8.2 | 19.5 |
| Over Nikolaev (35 km from Lvov) | 454 | 35.2 | 27.4 | 13.6 | 23.8 |

We should note first of all that on the day of investigation the direction of the wind coincided with the direction of flight of the plane and this enabled us to assess the effect of Lvov on the degree of bacterial contamination of the air at different distances from the town.

As we see from the data in the table, the greatest numbers of microbes – 1176 bacteria per $m^3$ of air – were found at a height of 250 m over the town. At a distance of 10 km from Lvov the bacterial content of the air was almost halved – to 628 per $m^3$. Their numbers hardly changed at all as the plane passed over the large forest area (662 per $m^3$ of air).

Later investigations of the air at the same height over the district centers Bobrka and Nikolaev showed that the microbial content of the air over them was almost the same – 434 and 454 per $m^3$, respectively. Approximately the same results were obtained when the airplane flew over the same route on the following day.

Thus, at a height of 250 m the degree of bacterial contamination of the air above Lvov was 2.5 times higher than over the lightly populated towns of Bobrka and Nikolaev, and at a distance of 10 km from Lvov the bacterial content of the air was almost halved. Taking into consideration the fact that the direction of the airplane and the wind coincided, and also that the bacterial contamination of the air was 1.5 times higher over the fields and forests than over the smaller towns, we can infer that Lvov affected the degree of bacterial contamination of the air over a horizontal distance of approximately 10-15 km.

Significant differences were discovered in a study of the qualitative composition of the microflora. In our previous investigations we found that the main forms of bacteria constituting the natural biological background of the upper atmosphere were sporing bacteria and fungi (Kishko [1959 a, b]).

We found that at a height of 250 m over Lvov pigment-formers predominated (47.6% of isolated microflora), sporing forms of bacteria were less numerous (31.6%), fungi were still fewer (14.7%), and there were very few (6.1%) colorless forms of microbes. Approximately the same proportional composition of the aeromicroflora was found in the air over Bobrka and Nikolaev. However, the air at the same height over fields and large stretches of forest had specific features. For instance, in air over fields the numbers of pigment-forming bacteria were reduced to 21.9%, and there was a marked increase in the number of sporing aerobes (up to 51.6%). The air above the forest was heavily laden with fungi (50% of isolated microflora), but pigmented forms of bacteria comprised only 17.9% of all the microbes.

In view of this, we can infer that at a height of 250 m over the town the features of "town" air are still retained. This point is illustrated by the 1957 results which we give for comparison in Table 2 (mean results of six to eight investigations of air of temperate continental latitudes).

As Table 2 reveals, the town air was heavily laden with pigmented forms of bacteria (62.9% of the isolated aeromicroflora) and contained very few fungi (1.6%). The atmosphere of the control point, which was outside the town and surrounded by forest, contained only about a third as many bacteria as in the town; here the numbers of fungi were much higher and constituted 11%, whereas the pigment-formers, on the other hand, were much fewer, comprising 48.7%.

At a height of 500 m the numbers of pigment-forming bacteria, as Table 2 shows, were still fewer, comprising 40.2% over the town, whereas outside they constituted 30% of the isolated aerial microorganisms. The numbers of sporing aerobes were increased – to 34.4 and 24%, respectively. The relative numbers of fungi were more than ten times greater over the town, and almost four times greater outside the town. At a height of 4000 m, however, no significant differences were found between the aerial microflora over the town and outside it.

It should be noted that the qualitative composition of the aerial microflora at this height differed radically from that of the town air. Whereas in the low layer of town air numerous pigmented forms of bacteria and very few fungi were isolated, in the upper layers of air sporing forms of bacteria and fungi predominated.

Thus, in the vertical direction Lvov affected the degree of bacterial contamination of the air up to 4000 m.

The studies of the zonal distribution of bacteria in the district of Nikolaev were of interest. Air samples for bacteriological investigation were taken between 7.30 AM and 9.30 AM on April 18, 1959, at different heights over Nikolaev within a radius of 1 km at distances of 7 and 14 km on the windward and leeward sides. The investigations were carried out in clear, cloudless weather after rain during the circulation of sea air of temperate continental latitudes.

The data obtained are given in Table 3.

TABLE 2

Microflora of Air over Lvov and Surrounding Areas

| Point of investigation | Microorganisms in 1 $m^3$ of air | Composition of microorganisms, % | | | |
|---|---|---|---|---|---|
| | | pigmented | sporing | colorless | fungi |
| | Height 1.5 m | | | | |
| Lvov | 4774 | 62.9 | 19.0 | 16.5 | 1.6 |
| L. Komsomol'skoe (5 km from Lvov) | 1719 | 48.7 | 19.5 | 20.8 | 11.0 |
| | Height 500 m | | | | |
| Over town | 632 | 40.2 | 34.4 | 6.5 | 18.9 |
| Outside town (7-10 km) | 360 | 30.0 | 24.4 | 4.8 | 40.8 |
| | Height 4000 m | | | | |
| Over town | 79 | 13.9 | 33.0 | 5.0 | 48.1 |
| Outside town (7-10 km) | 70 | 11.4 | 38.6 | 1.5 | 48.5 |

TABLE 3

Microbial Content of Air at Different Heights in Nikolaev District

| Height investigated, m | Wind speed, m/sec | Air temperature, C | Microorganisms in 1 $m^3$ of air | | | | |
|---|---|---|---|---|---|---|---|
| | | | windward side | | over town | leeward side | |
| | | | 14 km | 7 km | | 7 km | 14 km |
| 400 | 3.0 | 11.0 | 120 | 120 | 120 | 168 | 193 |
| 1000 | 7.0 | 10.0 | 36 | 60 | 204 | 264 | 132 |
| 2500 | 10.0 | 2.0 | 0 | 120 | 36 | 80 | 96 |

As the data in Table 3 show, the microbial content of the air at height 400 m above Nikolaev and on its windward side was the same (120 bacteria per $m^3$ of air). On the leeward side they were a little higher (168 and 192 per $m^3$ at distances 7 and 14 km, respectively), the wind speed being 3.0 m/sec.

With ascent into the atmosphere the wind speed increased. At a height of 1000 m it was 7.0 m/sec, and at a height of 2500 m it was 10 m/sec. In correspondence with this the zonal distribution of the aerial microflora was altered.

At a height of 1000 m the microbial content over the town (204 per $m^3$ of air) and on the leeward side (132 and 264 per $m^3$ of air) increased a little, but their numbers on the windward side were reduced by a factor of 3-6. At a height of 2500 m, however, the microbial content over the town on the windward and leeward sides was low.

Thus, the effect of Nikolaev on the degree of bacterial contamination of the upper air was much less pronounced in comparison with Lvov. As Tomson [1958] indicated, the formation of convection currents is greatly enhanced when the difference between the soil and air temperatures increases. Aerial convection currents laden with microbes in this case probably reached a height of 1000 m. The air temperature at the investigated heights varied from 11 to 2 C (the soil temperature was 14 C).

## SUMMARY

1. Lvov affects the degree of bacterial contamination of the upper air over a horizontal distance of up to 15 km.

2. Up to a height of 4000 m the aerial microflora over the town and outside it has specific features. Over the town pigment-formers are relatively numerous and fungal forms are few, while outside it the numbers of fungi and sporing aerobes are greatly increased, and the numbers of pigmented forms of bacteria are reduced. At a height of 4000 m the aeromicroflora is quantitatively and qualitatively similar.

3. Nikolaev affects the degree of bacterial contamination of the air up to only 1000 m.

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**All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.**


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# STUDY OF MICROORGANISMS OF MINUSINSK BASIN WITH REFERENCE TO GENESIS OF OZOCERITE-LIKE BITUMENS

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A fuller account of the role of microorganisms in the formation of the ozocerite-like bitumens of Minusinsk basin will be given in a paper devoted especially to this question.

From an examination of the published information, our own investigations, and the results of chemical studies of cultures of microorganisms developing on a mineral medium containing paraffin-base petroleum or paraffin (Mironov, Sokolova and Nikitina [1957]) we can conclude that in the transformation of paraffin-base petroleum into ozocerite-like bitumens aerobic microbiological processes are of paramount significance. Microorganisms of the genera Mycobacterium and Proactinomyces are of great importance in these processes.

The aim of the present investigation was a further study of the microorganisms utilizing paraffin-base petroleum occurring in outcropping bituminous rocks and in ozocerite-like bitumens. In addition, in order to discover the direction of the process of conversion of petroleum into bitumens we studied the behavior of the isolated microorganisms toward the paraffin-base petroleum of the Minusinsk basin and toward hydrocarbons of various structure.

## MATERIAL AND METHODS

The samples for investigation were collected at different points in the Minusinsk basin. We investigated seven samples of bituminous rocks from exposed Devonian strata, four samples of bitumens, and one sample of porphyrite containing bitumen. The sampling points and a characterization of the samples from the data of the Laboratory of Petroleum Genesis are given in Tables 1 and 2 (Mironov, Sokolova and Nikitina [1957]).

From our own results we have inserted in Tables 1 and 2 the names of the microorganisms and the numbers of the strains isolated from these samples.

For the experiments we used a pale paraffin-base petroleum of Bystryanka structure from well 2-P.

Petroleum of Bystryanka structure is distinguished by its low carbon content and high hydrogen content, which indicates the basic, limiting nature of the hydrocarbons in its composition. The petroleum is characterized also by its high solidification point and high content of solid hydrocarbons – about 10% (Sokolova, Mironov and Nikitina [1958]).

The material for the microbiological studies was prepared in the following way. The rocks and bitumens were first ground up in sterile conditions as indicated earlier (Shturm [1960]).

For the isolation of pure cultures we used a solid mineral medium of the following composition (g): $Na_2HPO_4$ -3.5; $KH_2PO_4$ -1.5; $NaNO_3$ -5.0; $FeSO_4$ -0.02; $Na_2SO_4$ -0.04; $CaCl_2$ -0.04; NaCl -10; tap water -1 liter; agar -2%; pH 7.2-7.4; microelements according to Fedorov [1951]. The only source of organic matter was the paraffin-base petroleum or paraffin.

The petroleum remaining after the experiment was separated from the microorganisms and from the liquid

medium in the following way. The culture fluid was filtered in batches through a double membrane preliminary ultrafilter at low pressure. Ultrafilters with smaller pore diameter were found to be unsuitable since the dense mass of microbial cells very soon clogged the pores. The filtrate was collected in small tubes contained inside a Bunsen flask. The filters were changed two or three times. The aqueous part of the culture passed into the filtrate, and the microbial cells and petroleum remained on the filter. The filters and flasks in which the cultures had been grown were then washed with small batches of hot benzene. The benzene solution of petroleum was filtered into a new dish inside a Bunsen flask. The contents of the dish were transferred to a separating funnel, the dish was again placed in the Bunsen flask and a new batch of benzene solution of petroleum filtered into it. This was repeated several times. For dissolving the petroleum we usually used about 100 ml of hot benzene.

TABLE 1

Microorganisms Isolated from Bituminous Rock Samples

| No. of sample | Sampling point | Horizon, division, series | Type of rock | Microorganisms |
|---|---|---|---|---|
| 41 | Near Lokshino village | $D_2$, Givetian, Saragash | Limestone | M. mucosum XIV, P. chrysogenum XIII |
| 41a | Near Uibat River | $D_2$, Givetian, Upper Élemorov | Limestones with adhering ozocerites Overlying fuel shales | M. globiforme, I,D M. coeliacum III M. mucosum XIV P, paraffinae V |
| 43 | Near Il'inki village | " " | Argillite overlying porous limestones | Bacterium sp. Mycobacterium sp. Proactinomyces sp. |
| 43a | Near Lokshino village | " " | Limestones with strong bituminous smell when split | Myxococcus ruber VI, VII P. corallinus Mycobacterium sp. |
| 44 | Near Il'inki village | $D_2$, Givetian, Upper Saragash | Porous limestones with strong bituminous smell | M. mucosum IV Penicillium miczynskii XIX |
| 44a | Near Lokshino village | $D_2$, Upper Givetian, Beisk | Organogenic limestone with bituminous smell | Myxococcus ruber VIII Pseudomonas sp. Mycobacterium sp. |
| 1 | Near Lake Ingol' | | Hard rock with adherent bitumen | Mycobacterium sp. Proactinomyces sp. |

The benzene solution of petroleum in the separating funnel was dried for 24 hr with anhydrous $Na_2SO_4$. It was then filtered through a smooth paper filter into a 250 ml distillation flask and the benzene was driven off (but not completely) on a water bath. From this flask the solution was transferred quantitatively into a previously weighed 50 ml flask and the benzene was driven off completely. The petroleuum was dried in vacuum and the flask was weighed.

All the preliminary filters with the bacterial deposit were extracted with benzene in a Soxhlet apparatus for 10 hr. The benzene was then driven off as described above and the quantity of extracted petroleum was determined. The quantity of petroleum obtained from the benzene solution in the separating funnel and from the benzene extract in the Soxhlet apparatus comprised the total quantity of residual petroleum in the culture.

The microorganisms were isolated by the usual plating method, various dilutions of rock suspension in sterile water or a weighed batch of ground bitumen (about 0.01 g) being used for inoculation. The weighed batch was introduced directly into sterile Petri dishes, and then melted sterile agar was poured in. The sterile petroleum

was either smeared directly on the surface of the congealed agar or was introduced with the melted medium into the tube.

TABLE 2

Microorganisms Isolated from Samples of Bitumens

| No. of Sample | Sampling point | Type of formation | Elementary composition, % | | | | | C/H | Ash | Microorganisms |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | C | H | S | N | O | | | |
| 131/52 | 2 km from Orositel'nyi station | Bitumen, dense asphaltite | 84.49 | 8.70 | 0.31 | 0.27 | 6.23 | 9.7 | 1.6 | M. flavum v. coeliacum III<br>Proactinomyces sp. |
| 130/52 | 99th km of Achinsk-Minusinsk road | Dense asphaltite | 80.43 | 10.55 | 0.25 | 1.68 | 7.09 | 7.6 | 0 | Mycobacterium sp.<br>Proactinomyces sp. |
| 138/52 | Baradzhul' | Porphyrite with bitumen (effusive rock) | 85.57 | 5.01 | Traces | Traces | 9.42 | 17.0 | 3.9 | Myxococcus ruber XII, XVII<br>P. notatum VI |
| 208/51 | " | Bitumen | | | | | | | | Myxococcus ruber X<br>Mycobacterium sp.<br>Proactinomyces actinoides XVI |

Further isolation of pure cultures was also conducted on an agar medium containing petroleum.

For a study of the morphological, cultural, and physiological properties of the microorganisms we used the organic media usually employed for diagnositic purposes and, in addition, we tested their behavior toward alcohols, organic acids, carbohydrates, paraffin, and paraffin-base petroleum.

To classify the microorganisms we used the keys of Krasil'nikov [1949] and Raper and Thom [1949].

## EXPERIMENTAL

An investigation of the colonies which developed on solid medium containing petroleum showed that all the rock samples indicated in Table 1 and the ozocerite-like bitumens (Table 2) contained pigmented microorganisms of the genus Mycobacterium and organisms of the genus Proactinomyces. Representatives of the genus Myxococcus were observed in rock samples Nos. 43a and 44a, and also in bitumen samples 138/52 and 208/51M. Representatives of the genus Penicillium were found in rock samples Nos. 41 and 44 and bitumen No. 138/52. Bacteriaceae were found only in asphaltite 130/50 and in one rock sample.

The poverty of flora of the family Bacteriaceae can probably be attributed to the specific features of the initial material and the conditions under which the cultures were isolated. A test of the behavior of the isolated bacteria toward paraffin-base petroleum showed that they consumed it to a slight extent.

A study of the morphological, cultural, and physiological properties of pure cultures of microorganisms isolated from the investigated samples showed that the isolated microorganisms were closely related to or identical with the following species: Mycobacterium globiforme (Conn) Krasil'nikov 1941; Mycobacterium flavum var. coeliacum Gray and Thornton 1928; Mycobacterium mucosum Krasil'nikov 1941; Myxococcus ruber Krasil'nikov 1941; Proactinomyces paraffinae Jensen 1918; Proactinomyces corallinus Jensen 1932; Proactinomyces actinoides Krasil'nikov 1941; Penicillium notatum Westling 1911; Penicillium chrysogenum Thom 1910; Penicillium miczynskii Zaleski 1941.

In Tables 1 and 2 we give a list of organisms and indicate the samples from which they were isolated. The Roman figures denote the strain numbers given to them in the investigation.

TABLE 3

Change of pH in Media Containing Different Sugars, Alcohols and Salts of Acids (initial pH in all cases 7.00)

| Name of micro-organism, strain \ Utilizable substrate | | Sugars | | | | | | | | | Acids | | | | Alcohols | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | arabinose | galactose | glucose | starch | lactose | levulose | maltose | sucrose | raffinose | acetic | citric | oxalic | butyric | ethanol | butanol | glycerol | mannitol |
| Myxococcus ruber | VI | 7.00 | 7.00 | 6.57 | 7.00 | 7.00 | 6.91 | 6.93 | 7.00 | 7.00 | 9.08 | 7.00 | 7.00 | 9.08 | 6.74 | 6.61 | 6.74 | 6.81 |
| | VII | 7.00 | 7.00 | 6.73 | 7.00 | 7.00 | 6.90 | 7.00 | 7.00 | 7.00 | 8.92 | 7.10 | 7.23 | 7.72 | 6.74 | 6.61 | 6.74 | 6.83 |
| | VIII | 7.00 | 7.00 | 6.88 | 6.84 | 7.00 | 6.71 | 6.89 | 7.00 | 7.00 | 8.90 | 7.02 | 7.16 | 7.15 | 6.97 | 7.00 | 6.93 | 6.95 |
| | XII | 7.00 | 7.00 | 6.52 | 7.00 | 7.00 | 6.85 | 6.88 | 6.84 | 7.00 | 9.06 | 7.00 | 7.18 | 7.78 | 6.77 | 6.64 | 6.81 | 6.93 |
| | XVII | — | — | 7.00 | 7.00 | 7.00 | — | 7.00 | 7.00 | — | — | — | — | — | — | — | — | — |
| | XVIII | 7.00 | 7.00 | 6.69 | 6.88 | 6.81 | 6.81 | 7.00 | 7.00 | 7.00 | 8.82 | 0.07 | 7.14 | 7.00 | 6.83 | 6.93 | 6.93 | 6.95 |
| Mycobacterium globiforme | I | 6.61 | 5.88 | 6.93 | 7.00 | 6.79 | 6.15 | 6.60 | 7.00 | 7.00 | 8.85 | 7.05 | 7.23 | 8.55 | 7.00 | 6.87 | 6.93 | 6.79 |
| | | 7.00 | 7.00 | 6.60 | 7.00 | 7.00 | 6.80 | 7.00 | 7.00 | 7.00 | 8.78 | 8.59 | 7.33 | — | 6.74 | 6.71 | 6.51 | 7.00 |
| M. flavum v. coeliacum | III | 7.00 | 5.58 | 4.54 | 6.43 | 6.42 | 5.23 | 5.88 | 5.75 | 7.00 | 9.06 | 9.09 | 7.18 | 9.01 | 6.39 | 6.74 | 6.34 | 4.97 |
| M. mucosum | IV | 6.93 | 7.00 | 5.27 | 7.00 | 7.00 | 6.68 | 6.83 | 4.93 | 7.00 | 8.71 | >9.00 | 7.19 | 9.34 | 6.57 | 6.46 | 5.74 | 6.15 |
| | XIV | 7.00 | 7.00 | 5.13 | 7.00 | 7.00 | 5.56 | 6.85 | 5.77 | 7.00 | 9.00 | 8.59 | 7.18 | 7.27 | 6.31 | 6.23 | 6.22 | 5.08 |
| Proactinomyces paraffinae | V | 7.00 | 7.00 | 5.72 | 7.00 | 7.00 | 6.21 | 6.90 | 6.89 | 7.00> | 9.00 | 7.12 | 7.18 | 9.32 | 7.00 | 7.00 | 6.05 | 6.03 |
| P. actinoides | XVI | 6.54 | 6.28 | 6.43 | 6.40 | 6.58 | 6.65 | 6.21 | 6.81 | 7.00 | 9.08 | 8.30 | 7.21 | 8.66 | 7.00 | 6.67 | 6.55 | 6.15 |
| P. corallinus | XI | — | — | 6.35 | 6.85 | 6.28 | — | 6.22 | 6.15 | — | — | — | — | — | — | — | — | — |
| Penicillium chrysogenum | XIII | 6.16 | 5.79 | 5.46 | 5.75 | — | 5.77 | 5.53 | 5.07 | 5.49 | 9.02 | 8.84 | 7.23 | 7.90 | 6.65 | 6.08 | 5.48 | 5.60 |
| Pen. notatum | II | 5.89 | 5.90 | 6.09 | 5.79 | 5.68 | 6.25 | 5.72 | 5.44 | 5.57 | 9.04 | 8.92 | 7.00 | 7.90 | 6.46 | 6.08 | 5.41 | 5.27 |
| Pen. myczynskii | XIX | 6.09 | 6.21 | 5.25 | 6.52 | 6.06 | 6.55 | 6.15 | 6.03 | 6.29 | 8.64 | 7.97 | 7.18 | 8.46 | 6.84 | 6.89 | 6.36 | 5.98 |

Note: —Indicates no determination made.

TABLE 4

Consumption of Paraffin-Base Petroleum by Microorganisms

| No. of culture | Duration of experiment, days | Name of microorganism | Petroleum added, g | Residue | | Loss | | Consumption with allowance for loss in control | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | g | % | g | % | g | % |
| 138b | 145 | Mycobact. mucosum | 2.2150 | 0.9992 | 45.11 | 1.2258 | 54.89 | 0.9110 | 41.66 |
| 140 | | Proact. paraffinae | 2.2942 | 0.9695 | 42.30 | 1.3247 | 54.69 | 1.0099 | 41.46 |
| 142 | | Nonsporing rod | 2.3417 | 2.1726 | 93.01 | 0.1691 | 6.94 | 0 | 0 |
| 145 | | Mycobact. flavum v. coeliacum | 2.1663 | 1.8754 | 83.55 | 0.3615 | 16.45 | 0.0417 | 3.22 |
| 153 | | Mixture of above cultures | 2.2586 | 1.2848 | 51.91 | 0.9734 | 43.14 | 0.6590 | 29.86 |
| | | Control* | 2.3360 | 2.0230 | 87.77 | 0.3148 | 13.23 | — | — |
| 147 | 275 | Pen. notatum | 2.2612 | 1.5755 | 51.95 | 0.9732 | 43.04 | 0.7280 | 32.19 |
| 164 | | Myxococcus ruber I | 2.1421 | 1.8177 | 84.64 | 0.3294 | 15.36 | 0.0347 | 1.61 |
| 156 | | Myxococcus ruber II | 2.3254 | 1.6248 | 69.83 | 0.7030 | 30.12 | 0.3588 | 15.35 |
| 162 | | Mixture | 2.2841 | 1.7716 | 79.27 | 0.4625 | 20.72 | 0.1553 | 6.96 |
| 163 | | Control | 2.2896 | 1.9236 | 84.01 | 0.3660 | 15.99 | — | — |

* Control – the same medium with petroleum, but with no microorganisms introduced. Mean of five determinations.

M. globiforme strain D differed from the species to which it was assigned in that it developed weakly on sugars, did not liquefy gelatin, did not coagulate or peptonize milk, and did not assimilate mannitol. On a medium containing petroleum it produced a water-soluble yellowish-green pigment.

To characterize the physiological properties of the isolated microorganisms we give the results of their behavior toward sugars, organic acids, and alcohols (Table 3).

The table reveals that microorganisms of the genus Myxococcus did not use arabinose, galactose, or raffinose. The weak acidification of the medium by some strains on starch, lactose, levulose, maltose and sucrose should probably be attributed to the inoculum. Strains VI, VII, XII and XVIII caused appreciable acidification of the medium on glucose.

All the cultures vigorously utilized sodium acetate and caused a shift of pH to the alkaline side. Only certain strains (VI, VII, X) used butyric acid. Not one of the strains utilized citric acid and probably none used oxalic acid. Appreciable differences between the strains were observed in their behavior toward alcohols. On ethanol and butanol, strains VI, VII and XII slightly acidified the medium, and VI and VII did likewise on glycerol, too. The other strains did not use these compounds. Not one of the Myxococcus strains developed on mannitol.

The various species of mycobacteria differed considerably from one another in their behavior toward carbon sources. M. globiforme (I) caused appreciable acidification of the medium on galactose and levulose, and slight acidification on arabinose and maltose. Glucose, starch, sucrose, and raffinose were not utilized at all. M. flavum v. coeliacum developed well and acidified the medium on galactose, glucose, maltose, lactose, sucrose and less well on starch and lactose. Arabinose and raffinose were not utilized. The two strains of M. mucosum did not differ significantly from one another in relation to sugars. They developed well and acidified the medium on glucose, levulose and sucrose. Arabinose, galactose, starch, lactose, and raffinose were not assimilated. Some differences between the various species of mycobacteria were observed in relation to acids and alcohols. M. globiforme developed well and alkalized the medium on acetic and butyric acids and did not develop on citric and oxalic. M. flavum v. coeliacum, like M. mucosum strain IV, failed to develop only on oxalic acid. On acetic, citric, and butyric acids there was a pronounced alkalization of the medium. M. mucosum strain XIV differed from strain IV in that it weakly alkalized the medium containing butyric acid.

M. globiforme I, as distinct from the other species, did not develop on any of the alcohols (ethanol, butanol, glycerol, or mannitol). M. flavum v. coeliacum and M. mucosum strains IV and XIV developed on all the alcohols tested and acidified the medium to some extent.

P. corallinus XI acidified the medium containing glucose, lactose, maltose, or sucrose. This microorganism was not tested on the other media.

P. paraffinae developed well only on glucose, and less well on levulose. An acid shift of the pH in correspondence with the growth was observed in both cases. With the other sugars the pH did not change. The acids utilized were butyric and acetic, and the alcohols utilized were glycerol and mannitol.

P. actinoides was much more active. It more or less vigorously formed acids on all sugars with the exception of raffinose. It utilized citric acid in addition to acetic and butyric acid; it developed very weakly on oxalic acid. Among the alcohols it used mannitol and glycerol most actively, whereas butanol and ethanol were not utilized.

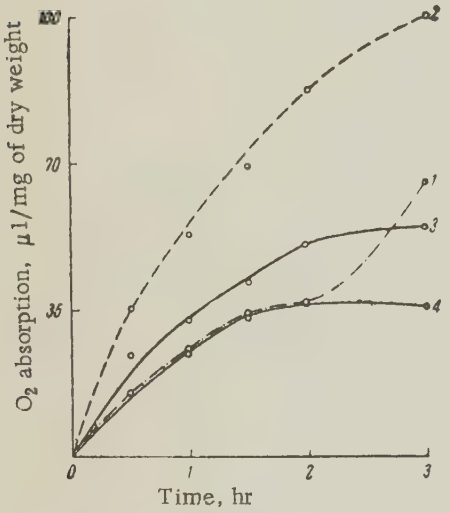


Fig. 1. Oxidation of hydrocarbons by Myxococcus ruber. 1) N-octane; 2) cyclohexane; 3) benzene; 4) endogenous respiration. Designations in Figs. 2-4 as in Fig. 1.

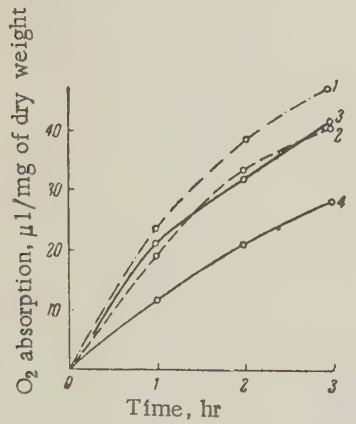


Fig. 2. Oxidation of hydrocarbons by Mycobacterium mucosum.

P. chrysogenum, P. notatum and P. miczynskii developed and acidified the media on all the investigated sugars. They all grew well on salts of acetic, citric, and butyric acid and caused an alkaline shift of the pH. With oxalic acid there was no change at all in the pH (P. notatum) or it was extremely slight.

P. chrysogenum and P. notatum developed well and acidified the medium on ethanol and butanol, and also on glycerol and mannitol. P. miczynskii used only glycerol and mannitol.

Further experiments aimed at discovering the behavior of the isolated microorganisms toward petroleum (Table 4) showed that in the first series of experiments P. paraffinae used up the greatest quantity of petroleum – about 1 g, or 41% – in 145 days. M. flavum v. coeliacum used about 0.4 g, or 3%, during the same time. The nonsporing rod did not develop on petroleum. It is of interest to note that a mixture of these cultures gave a lower percentage consumption of petroleum – 29%, or about 0.6 g (the table gives the mean value from two parallel cultures).

In the second series of experiments, which lasted 275 days, the greatest quantity of petroleum was used by P. notatum – about 0.7 g, or 32%. One of the strains of M. ruber (I) during the same time used only about 0.03 g, or 1.6%; the other (II) used 0.1 to 0.6 g, an average of about 15%. A mixture of these cultures was less active in utilizing petroleum – about 0.3 g, or 13.1%, in one of the parallel cultures, while in the other no development of microorganisms was observed.

From these data we see that in several cases there was a considerable variation in the quantity of petroleum consumed in parallel cultures, despite the fact that the same quantity of inoculum was added to the flasks.

Next, in connection with the posed problem, we ascertained how the microorganisms utilizing paraffin-base petroleum behaved toward different hydrocarbons of the paraffin and naphthene series, and also toward aromatic hydrocarbons. The experiments were conducted in a Warburg apparatus. The data obtained are shown in Figs. 1, 2, 3, and 4.

It is clear from these results that there were considerable differences between the four investigated microorganisms in their behavior toward hydrocarbons.

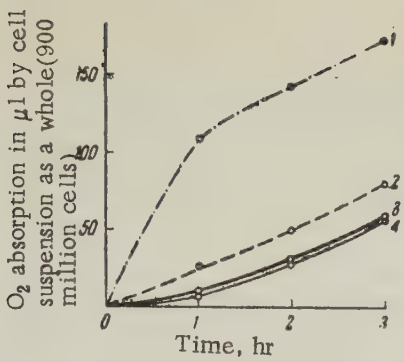


Fig. 3. Oxidation of hydrocarbons by Proactinomyces paraffinae.

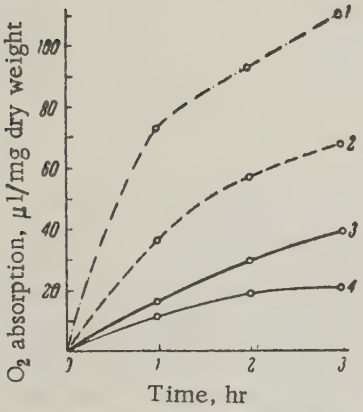


Fig. 4. Oxidation of hydrocarbons by Proactinomyces corallinus.

P. paraffinae vigorously oxidized n-octane, weakly oxidized cyclohexane, and did not utilize benzene. P. corallinus oxidized all three tested hydrocarbons; n-octane was oxidized most vigorously, cyclohexane much more weakly, and benzene still more weakly. M. mucosum most vigorously oxidized n-octane also; benzene and cyclohexane were oxidized at approximately the same rate. M. ruber oxidized cyclohexane most vigorously.

## SUMMARY

1. In ozocerite-like bitumens and in outcropping bituminous rocks of Devonian age in Minusinsk basin, microorganisms of the genera Myxococcus, Mycobacterium, Proactinomyces, Penicillium, and Pseudomonas occur.

2. Microorganisms insolated from samples of rocks and bitumens were similar in morphological, cultural, and physiological characters to the following species – Myxococcus ruber (four strains), Mycobacterium flavum v. coeliacum, Mycobacterium mucosum (two strains), Proactinomyces paraffinae, Proactinomyces corallinus, Proactinomyces actinoides, Mycobacterium globiforme, Penicillium notatum, Penicillium chrysogenum, and Penicillium miczynskii.

All these microorganisms possessed the ability to assimilate pale paraffin-base petroleum of Altai structure in aerobic conditions.

3. The different species assimilated hydrocarbons at different rates. M. ruber (strain VI) ovidized cyclohexane most vigorously, and octane less intensively. M. mucosum, P. paraffinae oxidized N-octane most rapidly, and cyclohexane less rapidly. The oxidation of benzene was extremely slow. P. paraffinae did not assimilate benzene at all.

4. The obtained results indicate the important role of aerobic microorganisms in the transformation of petroleum into the ozocerite-like bitumens of the Minusinsk basin.

5. We may surmise that in the process of transformation of petroleum the hydrocarbons of the paraffin series are first acted on by the microorganisms.

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# MICROBIOLOGICAL STUDIES OF CARPATHIAN SULFUR DEPOSITS

## III. HYDROGEN SULFIDE FORMATION IN YAZOV SULFUR FIELD

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The Yazov sulfur field is one of the largest deposits in the Carpathians and in its geological structure resembles other deposits of this region (Kuznetsov and Vetrov [1957]; Sokolov [1958]). In its hydrogeology, however, this field differs a little from Rozdol and Nemirov, which we investigated earlier (Ivanov [1960a, b]. As the illustrated geological section (Fig. 1) and schematic plan of the region of the field (Fig. 2) show, the water-bearing horizon of sulfur-bearing Ratinian limestones of the Yazov field is covered over its whole area by a water-impermeable layer of Sarmatian and Tortonian clay marls and never emerges under the Quaternary deposits. In view of this the surface water supply to the field takes place mainly through the Lower Tortonian limestones and sandstones which are extensive in this region and over a large area northeast of the field emerge under the water-bearing Quaternary deposits (Fig. 2). Water exchange between the Lower Tortonian rocks and the sulfur-bearing limestones is apparently effected through hydrogeological "windows" in the gypsums, through the broad strip of direct contact of these rocks in the southwest part of the field (Fig. 2) and, possibly, by filtration of the compressed waters of the Lower Tortonian horizon through the gypsum into the overlying sulfur-bearing rocks.

The two water-bearing horizons of the Yazov field (the horizon of Upper Tortonian sulfur-bearing limestones and the horizon of Lower Tortonian Lithothamnium limestones and sandstones) contain seam water with a high concentration of hydrogen sulfide (Tables 1 and 2). Since it is proposed to exploit the Yazov field by the open-cut method after a preliminary draining of the sulfur-bearing stratum, it was essential to study in what conditions and at what rate hydrogen sulfide was formed in the underground waters of this field. Hence, our investigations included a study of the distribution and activity of sulfate-reducing bacteria in the underground waters of the Yazov field at different times of the year.

During two expeditions (March-April and August, 1959) we investigated altogether more than 35 water samples from 11 self-filling wells sunk to the water-bearing Lower Tortonian deposits, and from eight wells draining water from the Upper Tortonian deposits. The positions of the investigated wells are shown in Fig. 2. An account of all the methods used in the field and laboratory work was given in one of the previous papers (Ivanov [1960a]) and hence we will not dwell on them here.

The results of investigations of the distribution of sulfate-reducing bacteria in the waters of the Lower Tortonian water-bearing horizon along a hydrogeological profile from the region of supply across the whole field (Fig. 2) are shown in Table 1.

The data given in Table 1 show that in the region of occurrence of weakly mineralized surface waters with high oxidation-reduction potential the numbers of these bacteria were low—200 cells or less per liter (drainage canal, wells 10G and 7G). Further along the seam their numbers increased a little, although in the waters of wells

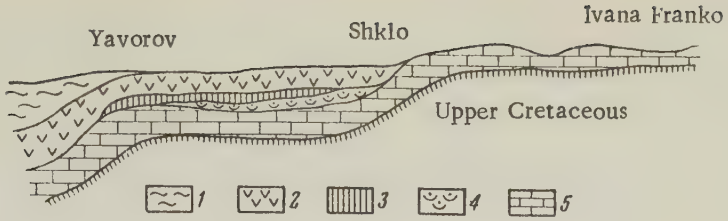


Fig. 1. Geological section through region of Yazov sulfur field (from Kuznetsov and Vetrov [1957]). 1) Quarternary deposits; 2) Sarmatian and Upper Tortonian clay marl layer overlying sulfur-bearing deposits; 3) Upper Tortonian sulfur-bearing limestones; 4) gypsum anhydrite layer; 5) Lower Tortonian Lithothamnium linestones.

TABLE 1

Chemical Composition of Waters of Lower Tortonian Water-Bearing Horizon of Yazov Sulfur Field and Numbers of Sulfate-Reducing Bacteria in These Waters

| Sampling point | Characteristics of water | | | | Number of bacteria |
|---|---|---|---|---|---|
| | dry residue mg/liter | $SO_4$, mg/liter | $H_2S$, mg/liter | $rH_2$ | |
| Drainage canal | 310 | 20 | 0 | 22.4 | <200 |
| Well 10 G | 186 | 18 | 0 | 23.7 | <200 |
| Well 7 G | 196 | 16 | 0 | 24.8 | 200 |
| Well 14 G | 236 | 15 | 0 | 19.0 | 1 000 |
| Well 1152 | 1825 | 1211 | 0 | 17.2 | 1 000 |
| Well 33 G | 2447 | 1472 | 94,6 | 11.7 | 1 000 |
| Well 21 B | 2620 | 1463 | 100.6 | 9.0 | 1 000 |
| Well 15 B | 2882 | 1546 | 117 5 | 10.5 | 10 000 |
| Well 24 G | 5892 | 296 | 515 0 | 7.8 | 10 000 |
| Well 38 G | 9936 | 12 | 169.5 | 7.8 | 10 000 |

TABLE 2

Rate of Sulfate Reduction, Hydrogen Sulfide Content and $rH_2$ of Underground Waters of Yazov Sulfur Field

| Well No. | April, 1959 | | | August, 1959 | | |
|---|---|---|---|---|---|---|
| | $rH_2$ | $H_2S$, mg/liter | Rate of $H_2S$ formation, mg/liter per day | $rH_2$ | $H_2S$, mg/liter | Rate of $H_2S$ formation, mg/liter per day |
| 70G | — | — | — | 14.1 | 9.4 | 0.539 |
| Spring 22 | 12.7 | 15.5 | <0.005 | 11.1 | 17.0 | 0.412 |
| 1241 | 10.8 | 67.6 | 0.075 | 9.6 | 63.3 | 1.350 |
| 1281 | 10.2 | 74.8 | 0.021 | 9.9 | 77.9 | 3.820 |
| K 5 | 10.0 | 80.0 | 0.065 | 8.6 | 81.4 | 1.059 |
| 1174 | 10.5 | 79.5 | 0.030 | 9.1 | 82.2 | 0.691 |
| 1285 | 9.6 | 113.1 | <0.005 | 9.0 | 116.9 | 0.823 |
| 1293 | 9.5 | 118.9 | 0.012 | 8.6 | 121.6 | 3.019 |
| 1297 | 10.6 | 117.5 | <0.005 | 8.8 | 142.1 | 3.612 |
| 12B | 9.0 | 80.6 | <0.005 | 9.0 | 83.6 | 2.030 |
| 15B | 10.5 | 117.5 | <0.005 | 8.6 | 121.7 | 1.052 |
| 38G | — | — | — | 7.8 | 169.5 | 1.601 |
| Mean | 10.34 | 86.5 | — | 9.23 | 90.8 | — |

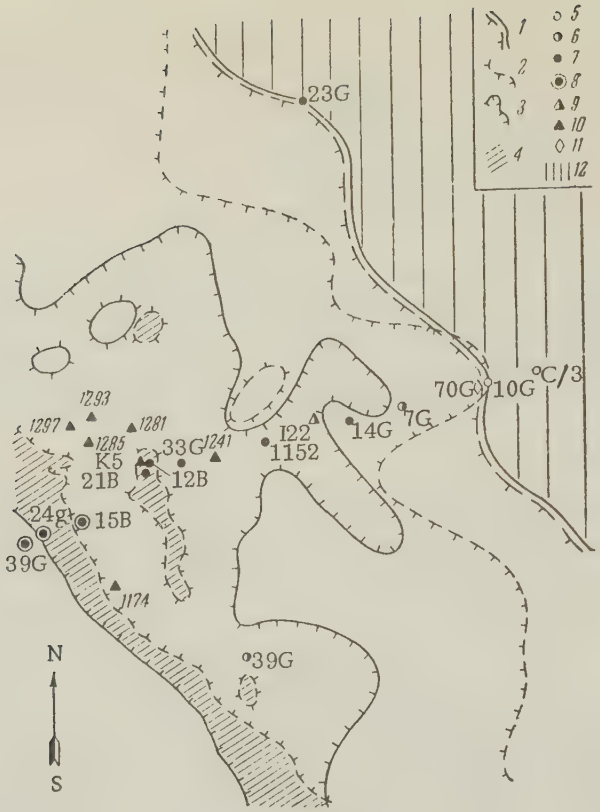


Fig. 2. Schematic plan of region of Yazov sulfur field. 1) Limit of extent of clay-marl layer; 2) limit of extent of gypsum anhydrite layer; 3) limit of extent of Upper Tortonian sulfur-bearing limestones; 4) region of direct contact of sulfur-bearing limestones and water-bearing Lower Tortonian Lithothamnium limestones; 5) wells draining water from Lower Tortonian deposits and in water of which no bacterial growth was found on inoculation of medium with 5 ml; 6) wells draining water from Lower Tortonian deposits and in water of which bacteria were found on inoculation of medium with 5 ml; 7) wells draining water from Lower Tortonian deposits and in water of which sulfate-reducing bacteria were found on inoculation of medium with 1 ml; 8) wells draining water from Lower Tortonian deposits and in water of which sulfate-reducing bacteria were found on inoculation of medium with 0.1 ml; 9) wells draining water from Upper Tortonian deposits and in water of which sulfate-reducing bacteria were found only on inoculation of medium with 5 ml; 10) wells draining water from Upper Tortonian deposits and in water of which sulfate-reducing bacteria were found on inoculation of medium with 1 ml; 11) wells draining water from Upper Tortonian deposits and in water of which sulfate-reducing bacteria were found on inoculation of medium with 0.1 ml; 12) region in which Lithothamnium limestones directly overlie Quaternary deposits—region of supply of surface waters to field.

14G and 1152, which had a fairly high oxidation—reduction potential, there was still probably no formation of hydrogen sulfide from sulfates, as was indicated by the absence of hydrogen sulfide in the water of these wells.

The greatest numbers of sulfate-reducing bacteria were found in hydrogen-sulfide waters with low potential (wells 15B, 24G, 38G). In the waters of the Lower Tortonian sulfur-bearing limestones, where the hydrogen sulfide content varied from 60 to 140 mg $H_2S$ per liter, and the $rH_2$ value lay between 8.6 and 10.8 (Table 2), the num-

bers of sulfate-reducing bacteria varied from 1000 to 10,000 cells per liter. Only in the water of spring 22 (Fig. 2), which had a low hydrogen sulfide content, did we find fewer bacteria, i.e., 200 cells per liter.

Thus, in the distribution of sulfate-reducing bacteria in the waters of Yazov field we observed the same main regularities as in the Nemirov and Rozdol fields which we investigated earlier: Sulfate-reducing bacteria occurred in all samples of hydrogen sulfide waters, and in almost all the waters containing no hydrogen sulfide, but their numbers were generally low. Growth in Shturm's modification of Tauson's medium was usually observed following inoculation with 5 or 1 ml of water and only in a few cases (wells 15B, 24G, 38G, 70G) did we find growth on inoculation with dilutions.

To investigate the rate of hydrogen sulfide formation in underground waters of Yazov field we conducted two series of experiments with labeled sulfate. The results of these experiments are given in Table 2.

The obtained data show that in the spring period the rate of the sulfate reduction process was extremely low and in not one of the investigated samples did it exceed 0.07 mg per liter per day. In the whole series of samples (wells 1285, 1297, 12B, 15B, and spring 22) the rate of hydrogen sulfide formation from sulfates was so low that even with the employment of the sensitive tracer method we could not establish any increase of hydrogen sulfide in the case of a 24 hr experiment. Since the sensitivity of the isotope method for the labeled sulfate concentrations which we used in the experiments on the Yazov field was approximately 0.005 mg $H_2S$ per liter per day, the rate of sulfate reduction in those samples where no increase of hydrogen sulfide was detected was shown to be less than 0.005 mg $H_2S$ per liter per day (Table 2).

Quite different values for the rate of formation of hydrogen sulfide from sulfates were obtained in the analyses of water samples from the same wells in the summer series of experiments. As the data of Table 2 show, the rate of sulfate reduction in the majority of samples varied around a value of 1 mg $H_2S$ per liter of water per day, and in several samples (wells 1281, 1293, 1287) exceeded 3 mg $H_2S$ per day. It is of interest to note that the absolute content of hydrogen sulfide in the water both in individual wells and in the field as a whole was higher in August than in April (Table 2).

Thus, both the direct experimental determination of the rate of sulfate reduction and the actual increase in the hydrogen sulfide content of the field waters in August indicate an intensification of the microbiological process of hydrogen sulfide formation from sulfates in the summer period.

It is also of interest to note that the $rH_2$ value in Yazov waters during the summer series of observations was a little lower than in spring (Table 2). In view of the fact that sulfate-reducing bacteria are obligate anaerobes we can surmise that it is the change in oxidation–reduction conditions toward a greater degree of anaerobism that leads to the stimulation of the activity of these bacteria in the summer.

As Fig. 2 shows, the majority of the wells which we investigated are situated in the central part of the field, in the region where waters with high hydrogen sulfide content occur. Only well 70G and spring 22 are situated fairly close to the supply region. In this connection it is interesting to note that it was in these wells that we found the lowest hydrogen sulfide concentration and the lowest values for the rate of sulfate reduction.

## SUMMARY

1. Sulfate-reducing bacteria are found in all the samples of hydrogen sulfide underground waters from the two water-bearing horizons of the Yazov sulfur field.

2. These bacteria are fairly frequent also in fresh hydrogen sulfide-free waters outside the boundary of the field.

3. Experiments with labeled sulfate show that hydrogen sulfide is being formed at present in both the water-bearing horizons of the field. The rate of this process in spring is low and is equal to 0.005-0.075 mg $H_2S$ per liter per day.

4. In summer the rate of hydrogen sulfide formation in the waters of the central part of the field increases to 1-3.8 mg per liter per day. The hydrogen sulfide content of the water is greater and the $rH_2$ is a little lower.

5. In waters with a low hydrogen sulfide content, which occur close to the supply region, the rate of sulfate reduction is much lower than in the central part of the field and amounts to only 0.5 mg per liter per day.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

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# ROLE OF BACTERIA IN OXIDATION OF SULFIDE ORES IN COPPER-NICKEL DEPOSITS OF KOLA PENINSULA

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The ability of thiobacteria to oxidize sulfides has been studied by Bryner, et al. [1954], Bryner and Anderson [1957], and Bryner and Jamerson [1958]. These authors showed that the bacteria, particularly Thiobacillus ferrooxidans, can play a definite part in the oxidation of a whole series of sulfide minerals, but they did not give sufficient consideration to the oxidation of ores in actual deposits. In view of this we have concentrated our attention in our studies on revealing the geochemical role of these organisms. Thus, we studied the occurrence of T. ferrooxidans in the Akhgal'sk mixed metal deposists in the Armenian SSR (Ivanov, Lyalikova and Kuznetsov [1958]), and also in several pyrite deposits in the Central Urals (Lyalikova [1960]). It was found that T. ferrooxidans plays an important role in the oxidation of the ores in these deposits, but it was still unclear: 1) if bacteria are involved in the oxidation of sulfide deposits in other climatic conditions, for instance in the conditions prevailing in the North, in the permafrost region; and 2) to what extent the oxidative processes depend on the mineralogical composition of the ores and, in particular, if copper-nickel sulfide ores are oxidized.

In this connection we undertook a study of the microflora of the copper-nickel deposits of Kola Peninsula, which differ from those investigated earlier both in mineralogical composition and in geographical location. It was of particular interest to determine if bacteria are involved in the oxidation of these deposits because, as Betekhtin indicated [1959], the processes of chemical change of sulfides proceed very slowly in the conditions of the cold polar climate.

Altogether we investigated four copper-nickel deposits. Two of them, Nittis-Kumuzh'e and the abandoned Sopcha field, are situated in the Monche Tundra in the center of Kola Peninsula and the other two, Kaula and Kammikivi, are situated in Pechenga District, Murmansk Region.

Nittis-Kumuzh'e is a deposit of the vein type. In mineralogical composition the ores consist of 60-85% pyrrhotite, 10-30% chalcopyrite, 10-15% pentlandite, and 5-10% magnetite. The thickness of the oxidized part of the deposits—the "iron cap"—varies in relation to the water-bearing capacity of the region and the thickness of the veins, and in certain cases reaches 150 m. The "iron cap" consists of limonites, and it also contains magnetite and geodes of native copper.

The mine is of the moderately flooded type. The source of supply of the underground water is the atmospheric precipitation, which percolates down to a depth of 150-180 m from the surface. The water penetrates most easily through the ore-bearing fissured zones, where the percolation of the water is accompanied by leaching of the sulfides (Chirkov [1939]).

According to the data of hydrogeologists the yearly inflow of water in 1958 was more than 1 million 800 $m^3$. The greatest inflow takes place in June, when the thawing of the snow occurs. The water in each pit is collected in reservoirs from which it is then removed from the mine. We made an examination of the reservoirs since this could give a general idea of the microflora of the mine. The number of T. ferrooxidans was counted by the dilution method in Leathen's mineral medium (Leathen [1951]) containing ferrous oxide.

TABLE 1

Examination of Reservoirs in Nittis-Kumuzh'e

| Pit No. | Depth of horizon, m | Reservoir | pH | No. of T. ferrooxidans cells per ml |
|---|---|---|---|---|
| 1 | 152 | North | 7.4 | 15 |
| | | South | 7.35 | 1 |
| 2 | 18 | North | 7.1 | 1 |
| | | South | 7.1 | 0 |
| 3 | 62 | North | 7.1 | Present |
| | | South | 7.1 | " |
| 4 | 18 | North | 7.4 | 0 |
| | | South | 7.5 | 0 |

We see from Table 1 that T. ferrooxidans was found only in very small numbers at depth 102 and 62 m. The small numbers of this organism were probably due to the high pH values.

The water from the reservoirs of pit 1 passes to the ore sorter, where it washes the pieces of ore and then passes along a wooden channel to the settling ponds. After washing the ore the water contains considerable quantities of nickel and copper and acquires a dirty grey color, since it contains many tiny fragments of ore. T. ferrooxidans, which was present in the water of pit 1, was found both immediately after the washing of the ore as well as in samples taken further downstream. It was absent only in the fourth sample, taken at a distance of about 1 km from the ore sorter, after the settling pond. Here the water was clear and the bacteria had obviously been removed by the settling ore particles. In addition to the reservoirs we also examined the water issuing from the galleries of pit 2. In the underground workings we examined the northern part of the field at the 102 m horizon. The results are given in Table 2. As the pH values given in the table show, the medium was acid in only two samples. These samples contained the greatest numbers of T. ferrooxidans, up to 1000 cells/ml, and in one of these samples, in water from gallery 1, T. thiooxidans was also present to the extent of 10 cells/ml.

TABLE 2

Occurrence of T. ferrooxidans in Nittis-Kumuzh'e Field

| Sampling points | | pH | Presence of ferrous oxide | Presence of ferric oxide | Number of T. ferrooxidans cells per ml |
|---|---|---|---|---|---|
| Drainage from galleries and water after ore sorter | Water from gallery 1 | 3.71 | + | + | 1000 |
| | Water from gallery 2 | 6.4 | – | + | 100 |
| | Water from gallery 10 | 7.1 | – | + | 10–100 |
| | Water from pit 2 at outlet | 6.8 | – | + | 10 |
| | Water from ore sorter after ore washing | 7.35 | – | – | + |
| | Water from ore sorter further downstream | 7.5 | – | – | + |
| | The same | 7.35 | – | – | + |
| | The same | 7.26 | – | – | + |
| | Water from ore sorter after settling pond | 7.2 | – | – | 0 |
| Underground mine waters | Drip from roof, pit 1, hor. 102, drift 9 | 6.5 | – | + | 10 |
| | Drip down timbering, pit 1, gallery 2, section 1 | 3.88 | + | + | 1000 |
| | Drip from roof, pit 1, vein 4, drift to north | 7.25 | – | – | + |
| | Water from ditch, pit 1, cross-cut to vein 12 | 6.1 | – | + | 1000–100 |
| | Oxidized ore containing cobalt, surface digging, vein 27 | Not determined | + | + | 10 |
| | Scrapings of oxidized ore, pit 1, vein 9, section 3 | " | + | + | + |
| | Scrapings of oxidized chalcopyrite, pit 1, drift 2 | " | – | – | 0 |
| | Scrapings of oxidized ore from gallery wall | " | + | + | 100 |

TABLE 3

Microbiological Investigation of Kaula Deposits

| Nature of samples | Number of samples examined | Number of samples containing T. ferrooxidans | | | | Number of samples containing T. thiooxidans | Number of samples containing Gallionella |
|---|---|---|---|---|---|---|---|
| | | total | 1 cell per g | 10 cells per g | 100 cells per g | | |
| Underground mine waters, pH 7.1-7.8 | 14 | 7 | 3 | 3 | 1 | 2 | 3 |
| Scrapings from ore | 2 | 2 | 1 | 1 | – | – | – |
| Samples of oxidized zone from open workings | 12 | 5 | 2 | 3 | – | 1 | – |

TABLE 4

Occurrence of T. ferrooxidans in Kammikivi Deposits

| Sampling point | pH | No. of T. ferrooxidans cells per ml |
|---|---|---|
| Hor. 300. Water from floor of gallery on way from hor. 300 to 320 | 7.44 | 10 |
| Hor. 320, water from roof of gallery | 8.1 | 0 |
| Hor. 320, drip from roof of gallery | 7.6 | 10 |
| Slope from hor. 320, water from gallery roof | 7.4 | 10 |
| Slope from hor. 320, ore scrapings | – | 0 |
| Hor. 340, second field drift, water from floor | 7.35 | 10–100 |
| Hor. 340, second field drift, water from well | 7.4 | 1 |
| Hor. 340, encrustations around well | 7.1 | 100 |
| Hor. 373, seventh section, water sample | 7.25 | 1 |
| Hor. 379, seventh section, ore sample | – | 0 |

Despite the fact that in other samples the reaction of the medium was almost neutral, T. ferrooxidans was absent in only two cases. It is possible that this reaction of the medium was partly due to the fact that we examined the deposits in July, soon after the spring floods, when the water in the workings was being renewed owing to the great inflow. According to old hydrogeological data for 1953-1954, the drainage was acid not only in the first gallery, but in the other galleries too. Since then the field had been heavily worked and the neutral reaction of the water from the galleries could possibly be also attributed to the lack of oxidizable material.

The examined dumps of pit 1 and 2 consisted mainly of empty rock, and ore was very scarce. From six samples sown in a medium for T. ferrooxidans, development was observed only in the case of one sample consisting of chalcopyrite with pyrrhotite.

In addition to the Nittis-Kumuzh'e field in the Monche Tundra we investigated the abondoned Sopcha mine, which has not been worked for 20 years. In its geological structure the Sopcha hill is similar to other elevations of the Monche Range and is composed of basic and ultrabasic rocks. The ores are disseminated and have a low nickel content, not exceeding 0.2-0.3%. Since the field has been closed up for a long time we could only take water samples issuing from four galleries. In all the galleries the pH of the water was 7.25, and inoculation into Leathen's medium revealed the absence of T. ferrooxidans.

In the Pechenga District of Murmansk Region we investigated the copper-nickel deposits of Kaula and Kammikivi.

Kaula is the largest deposit of the Pechenga ore field, the ores of which are derived from intrusions of basic and ultrabasic rocks. The ore body consists of a stratum-like deposit, the thickness of which varies from 1 to 30-40 m, and the length is 550 m; it lies predominantly in serpentinites in contact with sedimentary rocks—phyllites. The disseminated ores consist of pyrrhotite, pentlandite, chalcopyrite, and magnetite. The underground waters pass through cracks, the phyllites having the greatest water-bearing capacity (Eliseev [1952]; Gorbunov [1959]). According to the data taken from a Gipronikel' collection of technical information, the waters are discharged through a gallery into the river, the total flow at the mouth of the gallery being 16 liter/sec, and in spring it is approximately doubled.

We studied the Kaula deposits at horizons 6 and 7, situated 130-140 m above sea level. As Table 3 shows, the waters of the Kaula deposit are weakly alkaline. T. ferrooxidans was found in ten samples out of 15, and in two samples in which T. ferrooxidans was absent, T. thiooxidans was found. The neutral reaction and low temperature together with the high iron content created favorable conditions for the development of filamentous iron bacteria. Threads of Gallionella were found on the membrane filters.

Gallionella was earlier found in great numbers on slides set in gallery 10 of the Nittis-Kumuzh'e mine.

The Kaula deposit is also worked open-cut in addition to the underground working. We took samples from the oxidized zone, which was situated in the quarry at a depth of 15-20 m from the ground surface. The samples were coated with flowers of copper and contained a large amount of ferrous oxide. The samples were sown in Leathen's medium for T. ferrooxidans, in a hyposulfite-containing medium for the same organisms, and in Waksman's medium for T. thiooxidans.

Kammikivi belongs to an intrusive body sloping gently southwards, the lower part of which is composed of serpentinite and the upper of pyroxenite and gabbro. The ores contain about 3% nickel and 1.6% copper on the average.

The results of the examination are given in Table 4. T. thiooxidans was not found.

The results of the chemical and microbiological analyses indicate that at present no significant oxidation of sulfides is occurring in the Kola Peninsula copper-nickel deposits which we examined. In the mine waters of Nittis-Kumudzh'e, according to the hydrogeological reports for 1958, the mean quantity of nickel was about 15 mg/liter and of copper about 2 mg/liter, which is only about one-thousandth of the quantity of copper dissolved in the waters of some pyrite deposits, Degtyar deposit in the Central Urals, for instance. The role of T. ferrooxidans in the oxidation of the investigated deposits is obviously a minor one. This is due to a whole series of factors preventing the development of this organism.

First, the severe climatic conditions of the polar region have a great influence. The temperature in the underground workings of the Nittis deposits is always 4C. Second, the copper-nickel deposits contain no pyrites and are themselves genetically derived from basic and ultrabasic rocks and probably for this reason most of the mine waters have a neutral or slightly alkaline reaction.

The existence of T. ferrooxidans in these uncongenial conditions is obsviously due to its ability to create an acid medium in separate microzones, where the oxidation of the ores then takes place. As regards temperature, laboratory experiments with cultures isolated from these deposits showed their ability to develop at 4-5 C, although in these conditions the oxidation of iron was much slower than at 20-30 C.

The role of T. ferrooxidans in the investigated deposits varied. This role was obviously greatest in the vein deposits of Nittis-Kumuzh'e, where the waters have an acid pH and where this organism was found in 20 out of 25 investigated samples (excluding the dumps). The numbers of T. ferrooxidans in the waters of this field reached 1000 cells/ml.

In the waters of the closed Sopcha field, which has disseminated poor ores, T. ferrooxidans could not be found.

In Kaula and Kammikivi, which also contain disseminated ores, T. ferrooxidans was a little less widespread than in the Nittis-Kumuzh'e field and was found in smaller numbers.

SUMMARY

1. Four copper-nickel deposits of Kola Peninsula were examined for the presence of bacteria capable of oxidizing sulfide ores.

2. Hydrochemical evidence showed that oxidation of ores in the conditions of the Far North is weak.

3. Thiobacillus ferrooxidans and Thiobacillus thiooxidans were found in the Nittis-Kumuzh'e, Kaula and Kammikivi deposits in small numbers, usually not exceeding 10-100 cells per g.

4. The small numbers of these bacteria can probably be attributed to the alkaline reaction of the mine waters, which in turn depends on the mineralogical composition of the ores and the containing rocks.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

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# EFFECT OF CHEMICAL PREPRATIONS ON MICROBIOLOGICAL PROCESSES IN SILAGE AND ON ITS QUALITY

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Numerous investigations by our own and foreign authors have established that the ensiling of green material is inevitably accompanied by losses of nutrient material owing to the vital activity of putrefactive microorganisms and the respiration of the plant cells.

According to the data of Chesnokov and Zhabotinskii [1935, 1938], protein losses in spontaneous ensiling of clover reach 30%.

The studies of Nikolaeva, et al. [1957], showed that during the ensiling of alfalfa the loss of dry matter was 17.2%, and the loss of protein 72.8%.

In view of this the problem is to find improved methods of ensiling which will enable the obtention of silage of better quality with the minimum loss of nutrient substances.

Garder, Balin, Bogdanova, and Makarova [1935] devised a method of ensiling material by using a starter consisting of pure cultures of lactic acid bacteria.

TABLE 1

Number of Microorganisms on Ensilage of Clover with the Addition of Chemical Preparations (laboratory experiment)

| Experimental variant | Time of count, in days | Number of microorganisms (in 1000 per g) | | | | |
|---|---|---|---|---|---|---|
| | | lactic acid bacteria | putrefactive | coliform | butyric acid bacteria | yeasts |
| Clover before ensiling | — | 1 | 32 | 0.01 | 0.01 | 0 |
| Control silage | 2 | 150 000 | 19 000 | 1 000 000 | 1.0 | 0.03 |
| | 7 | 430 000 | 19 000 | — | 1.0 | 0.16 |
| | 20 | 240 000 | 2 000 | 1.0 | 1.0 | 0.06 |
| | 75 | 70 000 | 0.04 | 0 | 1.0 | 0 |
| Clover with VIK before ensiling | — | 0 | 0.14 | 0 | 0.01 | 0 |
| Silage with VIK | 2 | 8 | 0.23 | 0 | 0.01 | 0.36 |
| | 7 | 250 000 | 28 | — | 0.01 | — |
| | 20 | 120 000 | 14 | 0 | 0.01 | 52.0 |
| | 75 | 12 550 | 0 | 0 | 0.01 | 24.5 |
| Silage with S-2 | 2 | 8 | 22.5 | 1.0 | 0.01 | 28.0 |
| | 7 | 1 300 000 | 500.0 | 0 | 0.01 | 53.0 |
| | 20 | 50 000 | 0.06 | 0 | 0.01 | 0.21 |
| | 75 | 24 600 | 0.01 | 0 | 0.01 | 0 |
| Silage with AAZ | 2 | 1 650 | 1 850.0 | 1.0 | 1.0 | 4.0 |
| | 75 | 21 000 | 0.02 | 0 | 0.01 | 0 |

TABLE 2

Change in Microfloral Composition of Oat-Pea Silage Containing VIK after Eight Months of Ensilage (farm experiment)

| Experimental variant | Content of microorganisms (in 1000 per g) | | | | |
|---|---|---|---|---|---|
| | lactic acid bacteria | putrefactive | coliform | butyric acid bacteria | yeasts |
| Oat-pea mixture before ensiling | 850 | 8,550 | 100 | 100 | 14 |
| Control silage top | 18,950 | 9,950 | 0 | 1 | 0 |
| Control silage middle | — | 79 | 0 | 1 | 0 |
| Silage with VIK top | 6,800 | 6 | 0 | 0,01 | 0,06 |
| Silage with VIK middle | 17,850 | 15,5 | 0 | 0,01 | 0 |

TABLE 3

Effect of Various Chemical Preparations on Acid Composition of Clover Silage

| Experimental variant | Times of count, in days | pH | Total acidity, ml 0,1 N NaOH/100 g silage | Lactic acid and other nonvolatile acids | Free acids | | Bound acids | |
|---|---|---|---|---|---|---|---|---|
| | | | | | acetic | butyric | acetic | butyric |
| Clover before ensiling | — | — | 50 | — | — | — | — | — |
| Control silage | 2 | 5.2 | 90 | 0.68 | 0.08 | 0 | 0.16 | 0 |
| | 7 | 5.1 | 125 | 0.80 | 0.22 | 0 | 0.39 | 0.14 |
| | 20 | — | 160 | 0.94 | 0.33 | 0 | 0.48 | 0 |
| | 75 | 4.6 | 197.5 | 1.17 | 0.41 | 0 | 0.36 | 0 |
| Clover with AIV before ensiling | — | — | 150 | — | — | — | — | — |
| Silage with AIV | 2 | 4.0 | 145 | 1.26 | 0.03 | 0 | 0.09 | 0 |
| | 7 | 4.3 | 120 | 0.99 | 0.06 | 0 | 0.04 | 0 |
| | 20 | — | 170 | 1.29 | 0.13 | 0 | 0.08 | 0 |
| | 75 | 4.2 | 187.5 | 1.53 | 0.11 | 0 | 0.11 | 0 |
| Silage with S-2 | 2 | 4.2 | 120 | 1.05 | 0.02 | 0.01 | 0.05 | 0 |
| | 7 | 4.2 | 120 | 0.75 | 0.22 | 0 | | 0 |
| | 20 | — | 185 | 1.43 | 0.16 | 0 | 0.07 | 0 |
| | 75 | 4.2 | 215 | 1.43 | 0.34 | 0 | 0.01 | 0.02 |
| Silage with AAZ | 2 | 4.2 | 90 | 0.78 | 0.02 | 0.01 | 0.08 | 0 |
| | 75 | 4.2 | 257.5 | 1.75 | 0.38 | 0 | 0.06 | 0.02 |

Within recent years both in the USSR and abroad considerable interest has been manifested in the preservation of juicy forage by chemical preparations. The Finnish preparation AIV, which was developed by A. I. Virtanen and which contains hydrochloric and sulfuric acid, has become best known abroad.

In the Soviet Union in 1935, A. A. Zubrilin [1938] introduced a chemical preparation AAZ, consisting of a mixture of 10% solutions of hydrochloric acid and Glauber's salt in the ratio 7:3.

In 1955, two other mineral-acid preparations were suggested for ensiling fodders: 1) the preparation VIK of the All-Union Research Institute of Fodders, consisting of a mixture of 7 N solutions of sulfuric and hydrochloric acids in equal quantities; and 2) the preparation S-2 of the All-Union Research Institute of Horse Breeding, consisting of a mixture of sulfuric and hydrochloric acids.

In a comparison of the effectiveness of adding AIV and AAZ in the ensiling of clover, Berezovskii, et al. [1958], showed that the loss of dry matter in a control silage containing molasses amounted to 17%, but in the case of preservation with preparations AIV and AAZ it was 13.33% and 9.66%, respectively.

In an investigation of the preparations AAZ, AIV, and S-2 in the ensiling of green forage, Iskrov,et al. [1940], and Shmanenkov,et al. [1956],found that when mineral acids were added the loss of protein was reduced by a factor of 2-3.

As regards the accumulation of organic acids in green material preserved by chemical method there are contradictory data. In a study of the preparations AIV, S-2, AAZ, and VIK in the ensiling of corn cobs and alfalfa, Zakharchenko [1957] and Nikolaeva,et al. [1957],came to the conclusion that with chemical preservation the lactic acid content of the silage was reduced by a factor 1.5-2 in comparison with the usual ensilage. There was practically no butyric acid in this case, and acetic acid comprised only a very small percentage.

Berezovskii,et al. [1958],obtained opposite results in a determination of the organic acid content of clover silage prepared by different methods. In the control silage the lactic acid content constituted 2.83% of the absolutely dry matter, while with AIV and AAZ it was respectively 5.80 and 6.95%, i.e., 2-2.5 times more.

The aim of our investigation was to study the microbiological processes and biochemical changes occurring in the ensiling of green forage by the use of chemical preparations of mineral acids.

The experiments on the ensiling of protein rich fodder grasses with the employment of the preparations AAZ, AIV, S-2, and VIK were carried out in 1956-1958 in laboratory and farm conditions.

In the laboratory experiments we ensiled clover at the initial budding stage with the addition of preparations AIV, S-2, or AAZ. Ordinary (spontaneous) ensiling served as a control.

The preparations AIV and S-2, diluted 6.5 times with water, were added: in the first case the amount was 90 ml per 1 kg of grass, and in the second it was 100 ml. The preparation AAZ, according to Zubrilin's instructions, was added in an amount equal to 6.7% of the weight of the ensiled mass.

On the state farm "Detskosel'skii," Leningrad Region, we conducted an experiment on the preservation of an oat-pea mixture with preparation VIK in farm conditions. The ensiling was carried out in two cement pits with a capacity of 30 tons each. The oat-pea mixture was harvested by combine at the stage of milk ripeness of the oats. In one pit (experimental) 160 liter of VIK diluted with 960 liter of water was added to the silage mass, and in the second pit (control) the oat-pea mixture was ensiled without the addition of the preparation.

The microbiological analysis of the silage was conducted by the method of the All-Union Research Institute of Agricultural Microbiology. The organic acid content was investigated by Wiegner's method. At the same time we determined the sulfuric acid content of a water extract by the weight method after precipitation with a barium chloride solution, and we determined the hydrochloric acid content by titration with a 0.01 N solution of silver nitrate in the presence of potassium chromate.

The data characterizing the group composition of the microflora of green clover and the silage obtained at different times of ripening are given in Table 1.

When clover was ensiled with chemical preparations the lactic acid fermentation in the first few days was very weak. While the number of lactic acid bacteria in the control silage on the second day was 150 million per g, there was only 8,000 per g with preparations AIV and S-2. Judging from the rate of acid accumulation in milk and wort, the lactic acid bacteria in this period were in a depressed state. On the seventh day their multiplication was increased and they were only a little less numerous than the lactic acid bacteria of the control silage. The nonsporing proteolytic bacteria contained in the green material perished immediately after the addition of the chemicals or were present in small numbers (thousands of cells per g) in the silage only on the second day. The development of sporing putrefactive bacteria was also inhibited to a considerable extent. Although the butyric acid bacteria survived when the acids were added their numbers did not increase during the ripening of the silage. In the experimental clover silage yeasts developed at a greater rate and reached 52-53,000 as compared with 160 in the control. The results obtained in our experiment are in good agreement with those available on this question in the literature (see Leonov, Berezovskii, Sorokina and Prakhin [1958]; Cunningham and Smith [1940]).

Similar changes in the microfloral composition were observed when an oat-pea mixture was ensiled with the addition of VIK (Table 2).

Thus, as a result of these investigations we established that when the chemical preparations AIV, S-2, and VIK were used in the ensiling of green material consisting of clover or an oat-pea mixture the vital activity of

TABLE 4

Change in Acid Composition of Silage with Chemical Preparations S-2 and VIK Added

| Experimental variant | Times of count, in months | Total acidity in ml 0.1 N NaOH per 100 g silage | Percentage on wet weight basis | | | | |
|---|---|---|---|---|---|---|---|
| | | | lactic acid and other nonvolatile acids | free volatile acids | bound volatile acids | sulfuric acid | hydrochloric acid |
| **Laboratory experiment** | | | | | | | |
| Preparation S-2, sp. gr. 1.24 | – | – | – | – | – | 32.24 | 9.98 |
| Control clover silage | 3 | 120 | 0.43 | 0.46 | 0.20 | 0.07 | 0.17 |
| Clover silage with S-2 | 3 | 118 | 0.74 | 0.16 | 0.04 | 0.47 | 0.28 |
| **Farm experiment** | | | | | | | |
| Preparation VIK, sp. gr. 1.29 | – | – | – | – | – | 33.70 | 24.52 |
| Control oat-pea silage | 9 | 138 | 0.41 | 0.72 | – | 0.20 | – |
| Oat-pea silage with VIK | 9 | 213 | 1.25 | 0.44 | – | 0.37 | 0.46 |

the microorganisms did not cease completely. The development of the coliform, putrefactive, and butyric acid bacteria was mainly suppressed, whereas the lactic acid bacteria survived rather well.

A study of the dynamics of the accumulation of acids in clover silage containing chemical preparations gave the following results (Table 3).

In the spontaneous ensiling of clover the process of acid accumulation and reduction in hydrogen ion concentration was slow. In addition to lactic acid, a considerable amount of acetic acid in the free and bound state (0.77%) was formed in the silage.

When the green material was treated with AIV before ensiling its pH moved to 4.0, and its total acidity increased to 150 ml of 0.1 N solution per 100 g of silage. On the seventh day the titratable acidity of the silage was reduced to 120 ml owing to the fact that the lactic acid bacteria were in a depressed state and the mineral acids introduced with the preparation reacted with the bases of the vegetable mass. Later, with the intensification of lactic acid fermentation, the acidity of the silage increased and in the majority of cases exceeded the acidity of the control. No butyric acid at all was formed, and the content of acetic acid in the free and bound state was reduced by a factor 2-3.

In experiments on the ensiling of clover with S-2 and on oat-pea mixture with VIK we determined the sulfuric and hydrochloric acid content of the silage as well as the organic acid content (Table 4).

As Table 4 shows, in silage prepared by the chemical method, the greater part of the acidity consisted not of lactic acid, but of the mineral acids introduced with the preparation. In the ensiling of oat-pea mixture with VIK we found that sulfuric acid constituted 0.37%, hydrochloric acid 0.46%, and the sum of all the nonvolatile acids was 1.25%.

Table 5 gives the data on the change in the composition of nitrogenous substances for different methods of clover ensiling in laboratory conditions.

In the spontaneous ensiling of clover the active development of proteolytic coliform bacteria resulted in far-reaching breakdown of nitrogenous substances accompanied by an accumulation of up to 0.55% ammonia in the silage. When the chemical preparations AIV, S-2 and AAZ were added, the mineralization of the organic matter was much weaker, and the accumulation of ammonia in the silage was reduced by a factor 5-7. The lowest loss of protein was observed when clover was ensiled with AIV, where the protein loss did not exceed 4% of the initial content. In ensiling with preparations AAZ or S-2 the loss of protein was 16-17%, and in the control it was 25%.

TABLE 5

Content of Nitrogenous Substances on Ensiling of Clover with Chemical Preparations

| Material | Content, in % on absolute dry weight basis | |
|---|---|---|
| | protein | ammonia |
| Clover before ensiling | 21.07 | – |
| Control silage | 15.87 | 0.553 |
| Silage with AIV | 20.27 | 0.075 |
| Silage with S-2 | 17.56 | 0.120 |
| Silage with AAZ | 17.69 | 0.119 |

In the chemical ensiling of oat-pea mixture the loss of dry mattter was reduced by a factor 2.5. According to its organoleptic properties this silage was distinguished by the well preserved structure of the vegetable mass, pale green coloration and the presence of a pleasant fruit smell. Silage with VIK was eagerly eaten by cows at the rate of 20-25 kg per head per day.

It should be noted that the quality of clover silage prepared with the addition of S-2 or AAZ was almost as good as that obtained with AIV.

## SUMMARY

1. When clover or oat-pea mixture is ensiled with preparations AAZ, AIV, S-2, and VIK, which consist of mineral acids, the vital activity of microorganisms does not cease altogether. It is the development of the undesirable microflora – putrefactive, coliform, and butyric acid bacteria – which is mainly suppressed. The lactic acid bacteria are in a depressed state in the first two days, but later the process of lactic acid fermentation is fairly vigorous and differs little in intensity from that observed in spontaneous ensiling.

2. The addition of chemical preparations improves the quality of the silage as regards its organic acid composition. Butyric acid is absent, but acetic acid in the free and bound state accumulates in much smaller quantities.

3. The loss of dry matter in the preservation of an oat-pea mixture with VIK in comparison with ordinary ensiling is reduced by a factor of 2.5 owing to the better retention of protein and other nutrient substances.

4. A significant drawback of the chemical method of forage ensiling with the preparations AIV, S-2, and VIK is its awkwardness, the great expenditure of preparation and water per 1 ton of green material, and the high mineral acid content of the silage: sulfuric – 0.37-0.47%, and hydrochloric – 0.28-0.46%, on a wet weight basis.

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# OBTENTION OF METHANE AND ORGANIC FERTILIZERS BY FERMENTATION OF WOOD WASTE AND MANURE

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Several attempts have been made in the past to obtain organic acids and alcohol from wood waste, mainly by the use of thermophilic anaerobic cellulose-fermenting microorganisms. However, as Imshenetskii [1953] noted, so far neither alcohol nor organic acids have been obtained by the use of this group of microorganisms.

In the experiments of Olson, Peterson, and Scherrard [1937] wood sawdust was not fermented either after previous treatment with weak solutions of acids or alkalis, or after grinding in a ball mill.

Good fermentability of the wood was not obtained until the lignin content had been reduced to 1% as a result of oxidation. The reason for the nonfermentability of wood, in the opinion of the above investigators, was the presence of lignin in it, while its physical state was of no significance. Virtanen [1946], on the other hand, ascribes decisive importance in the fermentation of wood to its physical state and believes that cellulose is not chemically bound with lignin as a whole.

In his experiments up to 70% of the cellulose contained in pine, birch, and aspen dust was fermented.

In later investigations by Imshenetskii [1953], however, Virtanen's results were not confirmed. Without previous oxidation of the lignin the sawdust was not fermented at all. Thus, on this question the literature contains contradictory experimental data and different interpretations of these data.

The aim of our works was a little different. It was to find out if wood sawdust by itself or mixed with manure could be fermented by mesophilic anaerobic microflora to produce methane and organic fertilizers.

## EXPERIMENTAL METHOD AND RESULTS

The experiments were conducted in wide-necked 0.5 liter glass flasks.

The manure and sawdust were first mixed with an inoculum consisting of the fermented deposit of sewage waters which had ceased to give off appreciable quantities of gas.

The fermentation flasks were usually filled with substrate to not more than four-fifths of their volume.

The experiments were conducted at 90% humidity and at an ammonia nitrogen concentration of 1 g/kg of the mass.

In each fermentation flask we put 20 g of absolutely dry material for fermentation, 200 g of fermented deposit for inoculation of the medium with methane bacteria, and 160 g of tap water.

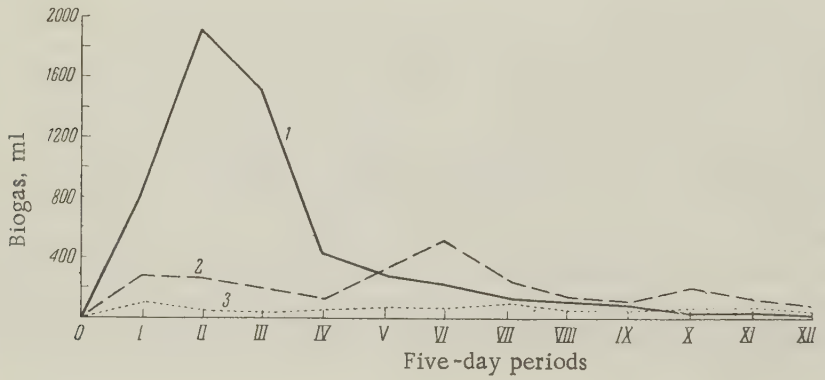
The flask was then completely closed with a rubber stopper, installed in an incubator at 32C, andconnected by means of a rubber tube to a gas collector filled with a saturated solution of common salt. Usually a vigorous evolution of gas began within the first 3-4 days. The gas evolved was measured from the amount of salt solution displaced from the gas-collecting flasks. Every experiment was conducted in two replicates.

The first experiment was carried out with sawdust and mixtures of it with manure and straw.

The results of the gas measurements are given in Table 1.

As the given data reveal, sawdust of coniferous or deciduous trees by itself underwent practically no methane fermentation over a period of 30 days, but in a mixture with cow and horse manure, or with straw, the sawdust of deciduous trees gave gas yields exceeding the yields of an equivalent amount of manure alone. Spruce sawdust, however, was not fermented even in a mixture with cow manure, but at the same time it had no adverse effect on the process of methane formation. Hence, the sawdust contained no toxic substances capable of slowing down or suppressing the fermentation process.

As we already mentioned, the experiments on methane fermentation of manure were usually conducted at 90% humidity of the fermentation material. All the early experiments with sawdust alone were also conducted at this humidity and they were always unsuccessful. When 40 g of aspen sawdust was put into a half-liter flask




Yield of biogas in fermentation of aspen and spruce sawdust in relation to substrate humidity. 1) Aspen sawdust, humidity 95%; 2) the same, 90%; 3) spruce sawdust, humidity 95%.

TABLE 1

Gas Yield (in ml) from Mesophilic Methane Fermentation of Sawdust Alone and in a Mixture with Manure of Various Farm Animals

| Material for fermentation, g | Five-day periods | | | | | | Total for 30 days | Per 1 g of dry matter |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | | |
| Aspen sawdust, 40 | 115 | 268 | 8 | 0 | 0 | 157 | 540 | 13 |
| Spruce sawdust, 40 | 132 | 89 | 76 | 104 | 84 | 165 | 650 | 16 |
| Aspen sawdust, 20 + rye straw, 20 | 1370 | 2842 | 2905 | 1630 | 1660 | 1212 | 11 619 | 290 |
| Rye straw, 40 | 1595 | 2175 | 2735 | 1730 | 1630 | 1049 | 10 914 | 272 |
| Aspen sawdust, 20 + cow manure, 20 | 668 | 2396 | 2080 | 2825 | 3542 | 1720 | 13 231 | 331 |
| Cow manure, 40 | 931 | 2067 | 2472 | 1745 | 1155 | 852 | 9222 | 230 |
| Spruce sawdust, 20 + cow manure, 20 | 507 | 994 | 1042 | 1264 | 447 | 505 | 4759 | 119 |
| Lime sawdust, 20 + horse manure, 20 | 269 | 1460 | 2150 | 1614 | 1525 | 900 | 7918 | 198 |
| Horse manure, 40 | 850 | 2425 | 1505 | 1068 | 827 | 376 | 7051 | 176 |

TABLE 2

Gas Yield in Methane Fermentation of Aspen and Spruce Sawdust in relation to Humidity

| Material for fermentation, g | Humidity % | Gas yield in each 5-day period, ml | | | | | | | | | | | | Gas yield | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | I | II | III | IV | V | VI | VII | VIII | IX | X | XI | XII | total, ml | per g of sawdust, ml | in relative units |
| Aspen sawdust, 10 | 95 | 820 | 1906 | 1510 | 412 | 217 | 160 | 120 | 70 | 60 | 36 | 28 | 30 | 5369 | 537 | 100 |
| Aspen sawdust, 10 | 90 | 277 | 265 | 108 | 106 | 308 | 539 | 249 | 119 | 99 | 181 | 124 | 58 | 2433 | 243 | 47.2 |
| Spruce sawdust, 10 | 95 | 80 | 56 | 40 | 58 | 76 | 81 | 104 | 47 | 50 | 40 | 60 | 52 | 744 | 74 | 13.8 |
| Spruce sawdust, 10 | 90 | 74 | 44 | 63 | 45 | 60 | 112 | 90 | 57 | 44 | 35 | 24 | 26 | 674 | 67 | 12.5 |

TABLE 3

Gas Yield in Methane Fermentation(32C) of Sawdust on Periodic Addition of Loads

| Indices | Duration of fermentation, days | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 22 | 14 | 14 | 18 | 18 | 19 | 23 | 22 | 31 | total |
| Sawdust introduced, g | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 100 |
| Biogas yield, ml | 4916 | 4552 | 4830 | 5288 | 4004 | 3972 | 4269 | 4278 | 3487 | 2782 | 42 378 |
| The same, per day | 246 | 207 | 345 | 378 | 222 | 221 | 225 | 186 | 158 | 89 | — |
| The same, per day, % | 100 | 84 | 140 | 154 | 90 | 90 | 91 | 76 | 64 | 37 | — |
| The same, per 1 g sawdust, ml | 492 | 445 | 492 | 529 | 400 | 397 | 427 | 428 | 349 | 278 | 424 |

we observed considerable swelling of the sawdust and an appreciable acidification of the fermentation material. In this case there was an accumulation of volatile fatty acids in quantities (more than 2000 mg/liter) which suppressed methane fermentation (Poch [1953]).

In later experiments fermentation of aspen and spruce sawdust was effected at 95 and 90% humidity. The results of these experiments are given in Table 2 and in the form of graphs (figure).

In the normal course of methane fermentation by mesophils the process usually terminates after 20-25 days. In this experiment the fermentation was continued for a longer time so that the utilization of the energy material would be as complete as possible. As the given data show, after 60 days of fermentation of 10 g of aspen sawdust at 95% humidity the yield of biogas* was 5369 ml, but it needed only a reduction of humidity by 5% to reduce the yield of gas to less than half as much (47.2) as in the preceding variant. Spruce sawdust, irrespective of humidity, gave insignificant yields of biogas, of no practical value. If we compare the yields of biogas during a shorter period – 20 days – we find that at 90% humidity the gas yield is only 18.4% of that obtained at 95% humidity.

The results of the conducted experiments refer to a single loading of the fermentation flasks with sawdust.

In order to follow the course of gas production when the quantity of sawdust added to the flasks was increased we conducted a long experiment. Into the fermentation flasks over a period of 201 days we added ten batches of 100 g of aspen sawdust. The time of introduction of the fresh material was usually determined from the rate of fermentation of the previous batch of sawdust. It lay between 14 and 23 days. The experiment was terminated when there was no liberation of gas in the last 15 days. The results of this experiment are given in Table 3.

* Here the term "biogas" means a mixture of gases consisting of approximately 60% methane and 40% $CO_2$.

TABLE 4

Composition of Gas Obtained in Mesophilic Methane Fermentation of Horse Manure and Wood Sawdust (volume %)

| Material | $CH_4$ | $CO_2$ | $O_2$ | $(N_2)$ |
|---|---|---|---|---|
| Aspen sawdust | 65.1 | 34.6 | 0.2 | 0.1 |
| Lime sawdust | 63.3 | 36.2 | 0.6 | 0.0 |
| Horse manure | 65.2 | 34.0 | 0.5 | 0.3 |

As the table shows the yield of biogas per 1 g of dry matter during the experiment varied in the range 278-529 ml. In this experiment an average of 424 ml of biogas was produced per g of sawdust. This greatly exceeded the gas yields from all the other agricultural waste materials which we tested earlier. After cessation of the gas yield, part of the contents of the fermentation flasks was analyzed for humidity, pH, volatile fatty acids and ammonia nitrogen. We established that ammonia nitrogen was completely absent from the medium and that there was a favorable ratio of the other indices for methane fermentation (pH 7.8, 150 mg of volatile acids per liter). The fact that fermentation was renewed at its previous rate after the addition of ammonia nitrogen to some of the flasks indicated that the lower yields of gas and the subsequent cessation of fermentation were due to a nitrogen deficiency in the fermentation material.

Since there was no loss at all from the hermetically sealed system we can surmise that the nitrogen was immobilized in the fermentation of the nitrogen-poor sawdust.

As we already noted, an important feature of wood sawdust, as distinct from other materials, as a raw material for fermentation is the high yield of fuel gas. When there is sufficient ammonia nitrogen in the fermentable medium the gas yield can be increased to 500 $m^3$ per ton of fermentable sawdust.

With such a yield of biogas the process of methane fermentation of sawdust will be profitable even at low external temperatures, since the expenditure of heat on heating of the methane apparatus is the same, irrespective of the biogas yield per unit of fermented material, and amounts to 25-30% of the quantity of biogas produced by the apparatus.

The quality of the biogas obtained from wood sawdust was not inferior to biogas from manure. For comparison we give the results of an analysis of the gas obtained from two samples of sawdust and from horse manure (Table 4).

The quality of the organic fertilizer obtained in this case is naturally of some importance for the farm. We know of the old experiments of Fedorov [1937], who obtained negative results in tests of fermented manure in plant pots, and Sauerlandt's later experiments [1951] conducted in fields in Germany and which gave positive results.

In this paper we cite some results of pot experiments in which we tested fermented and nonfermented sawdust as a fertilizer on the heavy loamy soil of the Central Experimental Station of the All-Union Institute of Fertilizers and Agronomic Soil Science in Borybino, Moscow Region. As test plants we used oats and sweet lupin. The experiment was conducted with soil previously treated with potassium phosphate fertilizer in pots containing 3 kg of soil. The sawdust in variants 4 and 5 was introduced to give a total nitrogen content of 0.1 g nitrogen per 1 kg soil, and in variant 6 the sawdust was introduced in pure form in an amount equivalent to the content in variants 4 and 5.

The results of the experiment are given in Table 5.

In this experiment fermented sawdust considerably increased the yield of oats, but fermented and nonfermented sawdust had practically no effect on the lupin yield. A mixture of nonfermented sawdust and inoculum applied to the oats had practically no effect, and sawdust alone had a pronounced adverse effect. Under the action of sawdust the assimilation of nitrogen by lupins was appreciably increased in every case.

Thus, wood sawdust fermented by mesophilic microorganisms becomes capable of increasing the yield and nitrogen assimilation even when applied 2-3 days before sowing of the spring crop, whereas nonfermented sawdust greatly reduces the yield. The adverse effect of nonfermented sawdust, as we might have expected, was not manifested in the case of lupin.

TABLE 5

Effect of Fermented and Nonfermented Wood Sawdust on Yield of Oats and Lupin and on Nitrogen Assimilation

| Fertilizer | Oats | | Sweet lupin | |
|---|---|---|---|---|
| | grain +straw, g | N assimilation, mg | grain +straw, g | N assimilation, mg |
| Soil with no fertilizer | 8.0 | 78 | 29.4 | 777 |
| PK | 9.8 | 82 | 35.9 | 884 |
| NPK | 25.0 | 292 | – | – |
| PK + fermented sawdust | 20.9 | 165 | 38.3 | 965 |
| PK + mixture of fermented sawdust and inoculum | 11.7 | 107 | 36.9 | 994 |
| PK + nonfermented sawdust | 2.04 | 25 | 34.4 | 914 |

## SUMMARY


1. Methane fermentation of sawdust of deciduous trees by mesophilic microflora is possible without previous acid hydrolysis.

2. The yields of biogas from sawdust of deciduous trees considerably exceed the biogas yields from manure and other agricultural wastes and reach 500 $m^3$ or more per ton of dry sawdust.

3. The rate of fermentation of wood sawdust is affected by the initial concentration of organic matter in the fermentable material. When the concentration of organic matter in the fermentable material is 10%, the fermentation of sawdust is extremely slow, which makes it useless for industry. When the initial concentration of organic matter is 5%, the process is normal with good yields of gas.

4. In chemical composition the biogas obtained by fermentation of sawdust and manure or of sawdust alone does not differ from the biogas obtained from fermentation of ordinary manure.

5. In the conditions of our laboratory experiments spruce sawdust was not fermented by anaerobic mesophilic microflora, but did not prevent its fermentation when mixed with manure and aspen sawdust.

6. Fermented sawdust with manure and sawdust fermented without manure considerably increased the yield and nitrogen assimilation of plants when applied to a spring grain crop. Nonfermented sawdust in the same conditions and in the same doses had a very adverse effect on the yield of oats. The fermented and nonfermented sawdust both promoted an increase of the nitrogen content of lupins.

7. Fermentation of sawdust mixed with manure or without manure can be used to obtain methane and organic fertilizers in agriculture.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# PREVENTION OF BIOLOGICAL ACIDITY REDUCTION DUE TO *Schizosaccharomyces acidodevorax* IN APPLE JUICES

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The observed biological reduction of acidity in apple juices and cider in various regions of the European USSR is mainly due to fission yeasts (Chalenko and Korsakova [1958, 1959, 1960]).

According to their morphological and physiological characters the new strains which we discovered and studied belonged to *Schizosaccharomyces acidodevorax* (1946). In this paper we give the results of investigations of the effect of mixed cultures and hydrogen peroxide on the process of acidity reduction during the fermentation of apple juice at different temperatures.

## Fermentation of Apple Juice by Mixed Cultures at Temperature 15-17 C

For the experiments we used pure cultures of *Saccharomyces vini* (ellipsoideus) strain Yablochnaya No. 7, Torulopsis strain Colville No. 21, and *Hansenula apiculata* strains Antonovka No. 27 and Antonovka No. 1. To these we added various numbers of cells of *S. acidodevorax* strain Voronezhskii No. 10. The apple juice, obtained from the "Mikhailovskii pereval" state farm of Krasnodar Trust, was poured into 50 and 150 ml flasks and sterilized twice for 30 min each time.

The juice had a titratable acidity of 8.25 g/liter calculated as malic acid, a saccharinity of 9.4%, specific gravity 1.042, and pH 3.3.

The experiments were conducted in two replicates.

After the introduction of a three-day-old growth of yeast cultures, some of the flasks were closed with fermentation fitments and were used for determining the fermentative ability from the loss of carbon dioxide. Others were closed with cotton stoppers and were used for observing the course of acidity reduction.

The results of the fermentation experiments and analysis of the cider are given in Table 1.

As Table 1 shows, fermentation by pure and mixed cultures at 15-17C continued for 23-31 days.

Here 1.92 to 2.15 g of carbon dioxide was evolved and 4.0 to 4.4 vol. % of alcohol was formed, and at the same time we observed a comparatively slight loss of acidity of the apple juice in all variants of the experiment according to the quantity of added pure culture of cider and deacidifying yeasts.

The same results for acidity reduction were obtained when the juice was fermented by *Hansenula apiculata* (strain No. 1) in quantity 2%.

The results of observations on the course of acidity reduction at 15-16C are given in Table 2.

TABLE 1

Lowering of Acidity of Cider by Schizosaccharomyces acidodevorax

| Yeasts | Duration of fermentation, days | $CO_2$ per 50 ml juice, g | Results of analysis after fermentation | | |
|---|---|---|---|---|---|
| | | | titratable acidity in g of malic acid per liter | Alcohol, vol. % | pH |
| Sacch. vini 2% (control) | 23 | 1.98 | 8.0 | 4.1 | — |
| Sacch. vini 2% + Schiz. acidodevorax 0.1% | 23<br>23 | 2.03<br>1.99 | 7.8<br>7.7 | 4.2<br>4.2 | 3.40<br>3.40 |
| Sacch. vini 2% + Schiz. acidodevorax 0.5% | 23<br>23 | 1.97<br>2.01 | 6.7<br>6.6 | 4.1<br>4.2 | 3.38<br>3.45 |
| Sacch. vini 2%+Hans. apicul 1.5%+ +Schiz. acidodevorax 0.1% | 28<br>28 | 1.99<br>1.97 | 7.3<br>7.3 | 4.2<br>4.1 | 3.31<br>3.31 |
| Sacch. vini 0.5%+Hans. apicul 1.5%+ +Schiz. acidodevorax 0.5% | 31<br>31 | 2.04<br>2.06 | 6.6<br>6.7 | 4.2<br>4.3 | 3.48<br>3.46 |
| Sacch. vini 0.5%+Torulopsis + 1.5% Schiz. acidodevorax 0.1% | 28<br>26 | 2.14<br>1.99 | 6.6<br>7.1 | 4.4<br>4.2 | 3.40<br>3.50 |
| Sacch. vini 0.5%+Torulopsis 1.5%+ +Schiz. acidodevorax 0.5% | 26<br>28 | 1.97<br>2.15 | 6.4<br>6.4 | 4.1<br>4.4 | 3.52<br>3.57 |
| Hans. apicul, (strain № 1) 2% +Schiz. acidodevorax 0.1% | 26<br>28 | 1.94<br>1.99 | 7.7<br>7.8 | 4.0<br>4.2 | 3.40<br>3.70 |
| Hans. apicul. (strain № 1) 2%+Schiz. acidodevorax 0.5% | 28<br>28 | 1.92<br>2.02 | 6.7<br>6.6 | 4.0<br>4.2 | 3.50<br>3.55 |

TABLE 2

Course of Acidity Reduction in Cider at 15-16° (initial titratable acidity 8.25 g/liter calculated as malic acid; pH before experiment 3.3)

| Time of expt., days | Temperature, C | Sacch. vini 2% | Sacch. vini 2%+ Schiz. acidodevorax 0.1% | Sacch. vini 2%+ +Schiz. acidodevorax 0.1% | Sacch. vini 0.5%+ +Hans. apiculata (strain №27) 1.5%+ +Schiz. acidodevorax 0.5% | Sacch. vini 0.5%+ +Hans. apiculata (strain № 27) 1.5%+Schiz. acidodevorax 0.5% | Sacch. vini 0.5%+ +Torulopsis 1.5%+Schiz. acidodevorax 0.1% | Sacch. vini 0.5%+ +Torulopsis 1.5%+Schiz. acidodevorax 0.5% | Hans. apiculata (strain №1) 2% Schiz. acidodevorax 0.1% | Hans. apiculata (strain №1) 2% +Schiz. acidodevorax 0.5% |
|---|---|---|---|---|---|---|---|---|---|---|
| 3 | 15 | 8.25 | 8.25 | 8.25 | 8.25 | 8.25 | 8.25 | 8.15 | 8.25 | 8.25 |
| 6 | 15 | — | 8.25 | 8.0 | 8.14 | 8.25 | 8.25 | 7.78 | 8.25 | 8.25 |
| 10 | 15 | — | 7.78 | 7.78 | 7.54 | 7.78 | 7.78 | 7.78 | 8.25 | 8.14 |
| 13 | 14 | — | 7.78 | 7.3 | 7.10 | 7.10 | 7.10 | 7.08 | 8.25 | 8.14 |
| 16 | 16 | — | 7.78 | 7.1 | 7.04 | 7.04 | 7.08 | 7.04 | 8.14 | 8.14 |
| 20 | 16 | 8.0 | 7.65 | 7.04 | 7.04 | 6.60 | 7.04 | 6.37 | 8.14 | 7.8 |

As in the previous experiment, there was hardly any loss of acidity in the case of fermentation by S. vini with the addition of 0.1% S. acidodevorax or by H. apiculata (strain No. 1) with S. acidodevorax (0.1-0.5%). In the other experiments there was a slight fall in acidity. As we know, a slight fall in acidity is caused by S. vini also, this being a normal process in the fermentation of apple juices.

On the conclusion of fermentation we made a microscopic examination of the yeast sediment in the experimental flasks. The microscopic examination showed that in flasks to which a 0.5% batch of deacidifying yeasts

TABLE 3

Acidity Reduction in Fermentation at 20-24 C

| Yeast | Duration of fermentation, days | $CO_2$ per 50 ml of juice, in g | Results of analysis after fermentation | | |
|---|---|---|---|---|---|
| | | | alcohol in vol.% | titratable acidity in g of malic acid | pH |
| S. vini 2% (control) | 12 | 2.04 | 4.2 | 7.3 | 3.4 |
| | 10 | 2.06 | 4.3 | 7.3 | 3.4 |
| S. acidodevorax 2% | 17 | 2.20 | 4.6 | 1.4 | 4.3 |
| | 17 | 2.24 | 4.6 | 1.3 | 4.3 |
| S. vini 0.5% + H. apiculata (strain No. 27) 1.5% + S. acidodevorax 0.1% | 12 | 2.08 | 4.4 | 6.8 | 3.5 |
| | 12 | 2.18 | 4.5 | 6.8 | 3.5 |
| S. vini 0.5% + H. apiculata (strain No. 27) 1.5% + S. acidodevoraz 0.5% | 12 | 2.20 | 4.6 | 5.6 | 3.5 |
| | 12 | 2.23 | 4.6 | 5.7 | 3.6 |
| S. vini 2% + S. acidodevorax 0.1% | 12 | 2.16 | 4.5 | 5.8 | 3.5 |
| | 12 | 2.20 | 4.6 | 5.7 | 3.6 |
| S. vini 2% + S. acidodevorax 0.5% | 12 | 2.17 | 4.5 | 3.5 | 3.6 |
| | 14 | 2.17 | 4.5 | 3.8 | 3.7 |

had been added, the sediment consisted mainly of pure cultures of budding yeast cells, and the deacidifying yeasts were present in very small numbers. Where a 0.1% batch of deacidifying yeasts had been added, there were only a few isolated cells in the yeast sediment.

## Fermentation of Apple Juice by Mixed Cultures at 20-24 C

We carried out investigations to reveal the process of acidity reduction during the fermentation of apple juice by mixed cultures at high temperatures (20-24 C).

The juice taken for the experiments had a titratable acidity of 7.5 g/liter calculated as malic acid, a pH of 3.34, and saccharinity 9.3%. The experiments were conducted in two replicates.

The results of the observations on fermentation and of the analysis of the fermented ciders at 20-24 C are shown in Table 3.

As Table 3 shows, the fermentation process lasted longer in the pure culture of S. acidodevorax. The quantity of alcohol formed varied from 4.2 to 4.6 vol %. The fermentation of sugars was accompanied by the destruction of malic acid. Apple juice fermented by S. vini suffered practically no loss of acidity, whereas in juice fermented by a pure culture of fission yeasts the loss of acidity was 6.1-6.2 g/liter, which corresponded to 82-83% of the initial.

Hence, the reduction of acidity in apple juices was much more rapid at higher temperature (20-24 C).

In mixed cultures, however, there was an insignificant fall in acidity. The results of observations on the course of acidity reduction at 20-24 C are shown in Table 4.

The data of Table 4 show that when apple juice was fermented by a pure culture of S. acidodevorax the destruction of malic acid was exceptionally rapid and by the fourth day the acidity was reduced to 1.9 g/liter, and later to 1.2 g/liter. When the apple juice was fermented by mixed cultures the fall of acidity was considerably

TABLE 4

| Time of expt, days | Temperature, C | Titratable acidity, g of malic acid per liter | | | | | |
|---|---|---|---|---|---|---|---|
| | | Sacch. vini 2% | Schiz. acidodevorax 2% | Sacch. vini 0.5%+Hans. apiculata (strain № 27) 1.5%+Schiz. acidodevorax 0.1% | Sacch. vini 0.5%+Hans. apiculata (strain № 27) 1.5%+Schiz. acidodevorax 0.5% | Sacch. vini 2%+Schiz. acidodevorax 0.1% | Sacch. vini 2%+Schiz. acidodevorax 0.5% |
| 1 | 23 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 | 7.5 |
| 2 | 23 | 7.5 | 3.7 | 7.5 | 7.1 | 7.5 | 7.1 |
| 4 | 23 | 7.5 | 1.9 | 7.3 | 7.1 | 7.3 | 7.1 |
| 8 | 22 | 7.5 | 1.2 | 6.8 | 6.8 | 6.6 | 4.7 |
| 11 | 20 | 7.5 | 1.2 | 6.6 | 6.4 | 6.4 | 4.5 |
| 14 | 22 | 7.5 | 1.2 | 6.6 | 5.7 | 5.9 | 3.5 |
| 17 | 24 | 7.5 | 1.2 | 6.6 | 5.6 | 5.9 | 3.1 |
| pH after expt. | | 3.34 | 4.4 | 3.5 | 3.6 | 3.5 | 4.0 |

TABLE 5

Effect of Hydrogen Peroxide on Development of Schizosaccharomyces acidodevorax

| Hydrogen peroxide concentration | Start of fermentation, in hr | Microscopic examination |
|---|---|---|
| Control | 40 | Cells elongate cylindrical and cylindrical |
| 0.02 | 40 | Short cylindrical |
| 0.04 | 64 | Cylindrical and round |
| 0.08 | 120–140 | Cells depressed |
| 0.16–9.24 | None | Cells dead |

retarded and occurred as in the previous experiment. The lowest fall in acidity was observed in juice fermented by mixed cultures of S. vini and H. apiculata (strain No. 27). A microscopic examination of the yeast sediment revealed that in these flasks, into which a 2% batch of pure or mixed cultures of budding yeast cells and a 0.5% batch of fission yeasts had been introduced, the yeast sediment consisted mainly of budding yeasts. The development of the fission yeasts was inhibited and they were present in small numbers. Where a 0.1% batch of fission yeasts had been added, only a few isolated cells were found in the yeast sediment.

## Effect of Hydrogen Peroxide on Vital Activity of Fission Yeasts

The aim of the experiments was to determine the lethal doses of hydrogen peroxide for fission yeasts. This might allow the use of hydrogen peroxide for sterilizing the juices and their subsequent fermentation by pure cultures of cider yeasts. A pure culture of S. acidodevorax No. 45 Maikopskaya was tested.

Into test tubes containing 10 ml of sterile apple juice we introduced a 0.5% batch of yeast cultures in the fermenting state (three-day-old culture) and then to these tubes we added a solution of hydrogen peroxide in the following amounts—0.02%, 0.04%, 0.08%, 0.16%, and 0.24%.

As a control we used apple juice containing a culture of S. acidodevorax No. 45 Maikopskaya and no hydrogen peroxide. The apple juice was placed in an incubator at 25–26 C. The results of the observations are given in Table 5.

As Table 5 shows, the lethal concentration of hydrogen peroxide for the fission yeasts was 0.16-0.24%. However, these doses imparted a very sour taste to the ciders and lowered the quality.

At the same time we conducted observations to reveal the effect of hydrogen peroxide on the vital activity of the cider yeasts Yablochnaya No. 7. We found that a hydrogen peroxide concentration of 0.16-0.24% was also lethal for the cider yeasts S. vini.

In a further experiment we employed smaller hydrogen peroxide concentrations (0.04-0.08%) to produce a temporary arrest of development of the fission yeasts and to create congenial conditions for the cider yeasts.

The juice was inoculated with a culture of S. acidodevorax No. 45 Maikopskaya.

TABLE 6

Effect of Hydrogen Peroxide on Fermentation with Fractionated Introduction of Yeasts to Juice

| Culture | Duration of fermentation, days | $CO_2$ per 50 ml juice, g | Results of analysis: titratable acidity, g malic acid per liter | pH | microscopic examination |
|---|---|---|---|---|---|
| Sacch. vini 2% | 12 | 1.75 | 8.0 | 3.2 | Yeast cells of S. vini type |
| | 7 | 1.81 | 8.0 | 3.2 | |
| Schiz. acidodevorax № 45 Maikopskaya 0.5% | 10 | 1.87 | 2.6 | 3.5 | Cells of oval and ovoid form, occasionally of the narrow short cylindrical Schizosaccharomyces type |
| | 7 | 1.95 | 2.1 | 3.85 | |
| Schiz. № 45 Maikopskaya 0.1% + $H_2O_2$ 0.04% + + Sacch. vini 2% | 8 | 1.56 | 7.9 | 3.36 | Yeast cells of S. vini type |
| | 8 | 1.48 | 7.9 | 3.36 | |
| | 8 | 1.54 | 7.9 | 3.36 | |
| Schiz. № 45 Maikopskaya 0.1% + $H_2O_2$ 0.08% + Sacch. vini 2% | 8 | 1.49 | 7.9 | 3.35 | The same |
| | 8 | 1.23 | 8.0 | 3.35 | |
| | 8 | 1.54 | 8.0 | 3.35 | |

For the experiment we used apple juice with acidity 8 g/liter and pH 3.25 from the Annenskoe wine factory. The filtered apple juice was poured into 50 ml flasks, the flasks were closed by cotton plugs and were sterilized by flowing steam for 20 min twice during the course of a day.

When the juice had cooled, a 0.1% batch of a four-day-old culture of fission yeasts and various doses of hydrogen peroxide were introduced, and after 24 hr a 2% batch of cider yeasts Yablochnoe No. 7 was added. The flasks were closed by fermentation fitments and were set up for fermentation at 26 C. The results of the observations on fermentation and an analysis of the fermented apple juice are given in Table 6.

Table 6 shows that fermentation in all the flasks was normal, i.e., it lasted eight days; fermentation set in on the second day. The titratable acidity after fermentation did not change and was the same as in the initial juice (7.9-8 g/liter). The hydrogen ion concentration was practically constant, too (pH 3.35).

In some of the experimental flasks the hydrogen peroxide after fermentation was completely decomposed, and in others traces were present (0.005%).

Hence, hydrogen peroxide in concentration 0.04% can be employed for the temporary inhibition or complete suppression of the development of fission yeasts and the creation of congenial conditions for the pure cultures of cider yeasts. However, the fermented apple juices acquire a slightly sour taste. Hence, the employment of hydrogen peroxide in industrial conditions will require a further development of the technology of application.

## SUMMARY

1. There is hardly any biological reduction of acidity when apple juices are fermented at low temperature (15-17 C) by pure cultures of cider yeasts added in 2% batches together with a 0.1% batch of Schizosaccharomyces acidodevorax.

2. The reduction in acidity when apple juices are fermented by mixed cultures of Saccharomyces vini and Hansenula apiculata at 15-17 C depends on the numbers of fission yeast cells. When the quantity of S. acidodevorax is increased to 0.5%, the acidity is reduced by 1.3-1.4 g/liter.

3. At 20-24 C the reduction of acidity in apple juices is greater, since the fission yeasts develop more rapidly than the wine yeasts. When the juice is fermented by mixed cultures containing H. apiculata a slight reduction of acidity (by 0.7 g/liter) is observed, since H. apiculata at the higher temperature develops more rapidly than the fission yeasts and displaces the latter.

4. The biological acidity reduction due to fission yeasts in the case of slight infection (up to 0.1%), as is most common in practice, can be prevented by the timely addition of cold-tolerant pure cultures of cider yeasts in batches of not less than 2% and the fermentation of the apple juice at lower temperature (15-17 C).

5. To prevent the reduction of acidity, hydrogen peroxide in concentration 0.04% can be employed for the inhibition or temporary suppression of the development of fission yeasts in apple juices. After 24 hr pure yeast cultures are added in a quantity not less than 2%. The use of hydrogen peroxide in industrial conditions requires further development of the technology of application.

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# USE OF NYSTATIN IN COMBATING MEAT MOLD

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One of the forms of spoilage of meat and meat products is that due to molds, which impart to the meat an unpleasant appearance and odor, thus lowering its market value. This is why it is so important to combat molds in meat-processing establishments, where there are congenial conditions (air temperature and humidity) for the growth of molds.

Among the proposed methods of preventing meat spoilage the preservation of meat in an atmosphere of 10--20% $CO_2$ is effective. However, the construction of special gastight chambers and careful control of the rate of gas supply and safety measures constitute a definite complication.

The studies on the use of ultraviolet radiation in the control of meat product spoilage due to bacteria and molds (Golovkin [1952]; Danilov [1947]; Matschullat and Günter [1958]) are of interest. Ultraviolet irradiation, however, does not completely exterminate mold spores (even in 24 hr at distance 25 cm).

An effective means of combating molds is disinfection of the rooms in which the meat is processed and stored. Among the agents suggested for this purpose the most promising is sodium oxydiphenolate (preparation F-5). Whitewashing of the rooms with this substance keeps the walls free of molds for 9-12 months (Dyklop [1952]).

Treatment of the surface of the product in addition to disinfection of the rooms would certainly greatly increase the effectiveness of mold prevention measures. However, the situation is complicated by the fact that it is exceedingly difficult to find a preparation suitable for destroying molds directly on the product. Such preparations must satisfy the following requirements: They must be harmless to the human organism, must not affect the color, odor, or quality of the product, and must act effectively on molds. Among the preparations meeting these requirements we can mention sorbic acid and vitamin $K_5$. Sorbic acid is used abroad for mold control on cheeses, marinades, confectionery, fruits, juices, and so on (Melnick and Vahlteich [1956]; Robinson and Hills [1959]; Rollin and Smith [1954]). Vitamin $K_5$ suppresses secondary fermentation in sweet wines, prevents mold growth on fungal sauce, jam, and other products and is ten times more effective than sorbic acid in the prevention of mold growth on the surface of cheese (Caspar and Jang [1957]; Stelle and Jang [1958]).

Within the last decade the antifungal antibiotic nystatin, which is used for the treatment of secondary mycoses (Tebyakina, et al. [1958]; Shorin and Gol'dberg [1957]; Limans, et al. [1955-1956]) has become widely known in medicine. Nystatin has little toxicity, is not easily absorbed by the intestine, and molds cannot become adapted to it. Hence, it can be used for preventing mold growth on food products also. Vacowitz and Pansy [1957] conducted experiments in which they submerged chicken wings and legs for 1-2 hr in a nystatin solution containing 5-10 ppm in combination with chlortetracycline (10 ppm). As a result the growth of yeasts and molds was prevented and there was observed an absence or significant reduction of yeasts and fungal odors after 13-15 days of storage of the meat at 5 C.

In the Antibiotics Laboratory of the All-Union Scientific Research Institute of the Meat Industry, methods of combating bacterial spoilage of meat by means of the antibiotic chlortetracycline (CTC) have been developed and have given positive results (Dyklop [1959]). However, when chlortetracycline was used, mold growth on meats was

TABLE 1

Fungicidal and Fungistatic Concentrations of Antimold Preparations (in μg/ml)

| Name of preparation | Test cultures | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Mucor. sr. | | Aspergillus sp. | | Penicillium sp. I | | Penicillium sp. II | |
| | fungistat. | fungicid. | fungistat. | fungicid. | fungi-stat. | fungi-cid. | fungi-stat. | fungi-cid. |
| Preparation K-25 | 0.4 | 15.6 | 1.9 | 1.9 | 7.8 | 15.6 | 31.2 | 31.2 |
| Arquad C | 3.9 | 31.25 | 31.2 | 31.25 | 3.9 | 3.9 | 3.9 | 7.8 |
| Diocide | 31.2 | 62.5 | 15.6 | 62.5 | 15.6 | 31.2 | 15.6 | 15.6 |
| Cetlpyridinium chloride | 7.8 | 7.8 | 31.2 | 31.25 | 15.6 | 15.6 | 3.9 | 3.9 |
| Katapin | 7.8 | 7.8 | 3.9 | 15.6 | 7.8 | 15.6 | 7.8 | 15.6 |
| Kationat 2-B | 15.6 | 15.6 | 15.6 | 62.5 | 7.8 | 7.8 | 7.8 | 7.8 |
| Nystatin (2900 units/mg) | 31.2 | | 250.0 | | 125.0 | | 62.5 | |
| Nystatin (3100 units/mg) | 62.5 | 125.0 | 125.0 | 500.0 | 125.0 | 250.0 | 31.2 | 250.0 |
| Vitamin $K_5$ | 46.8 | 93.7 | 93.7 | 187.5 | 46.8 | 46.8 | 93.7 | 187.5 |
| Arquad 2C | 39.0 | 156.2 | 156.2 | 312.5 | 78.1 | 156.2 | 39.0 | 39.0 |
| Sorbic acid (imported) | 312.5 | 2500.0 | 625.0 | 2500.0 | 312.5 | 625.0 | 312.5 | 625.0 |
| Sorbic acid (home-produced) | 625.0 | 2500.0 | 625.0 | 2500.0 | 312.5 | 625.0 | 312.5 | 625.0 |
| T-1 | 250.0 | 1000.0 | 500.0 | 500.0 | 500.0 | 500.0 | 250.0 | 500.0 |

Fig. 1. Chlortetracycline-treated meat heavily covered with yeasts and molds (14th day of storage in all figures).

not suppressed but, on the contrary, was even enhanced a little. This gave rise to the question of finding an antimold preparation suitable in combination with chlortetracycline for protecting meat from molds.

The aim of this work, which was carried out in 1959, was to find preparations effective against molds developing on meat and in chilled meat storage chambers. The work was conducted along two lines: 1) a determination of the fungistatic and fungicidal concentrations of preparations on pure cultures of molds; 2) a test of promising preparations on meat.

We tested 16 antimold preparations, including five antibiotics: nystatin (activity 2900 and 3100 units/mg), albofungin, griseofulvin and trichothecin, the last three in the form of culture fluids; seven quaternary ammonium compounds – Arquad C, Arquad 2C, Diocide, Katapin, Kationat 2-B, preparation T-1 and cetylpyridinium chloride; home-produced and imported sorbic acid; vitamin $K_5$ and preparation K-25, synthesized in VNIIKOP.

As test cultures of molds we used pure cultures which we isolated in the experiments: Mucor sp., Aspergillus sp., Penicillium sp. I and Penicillium sp. II, which occur most often on beef. The work with pure mold cultures was carried out by the serial dilution method (in Sabouraud's liquid medium). For ease of comparison of the various preparations their activity was expressed in the same units (μg/ml).

Table 1 gives the fungistatic and fungicidal concentrations obtained for the mentioned molds.

Table 1 shows that the preparations can be divided into three groups as regards fungistatic action.

Group 1, containing subtances with a fungistatic effect in the range 0.4 to 50 μg/ml, includes six preparations – K-25, Arquad C, Diocide, cetylpyridinium chloride, Katapin, and Kationat 2-B.

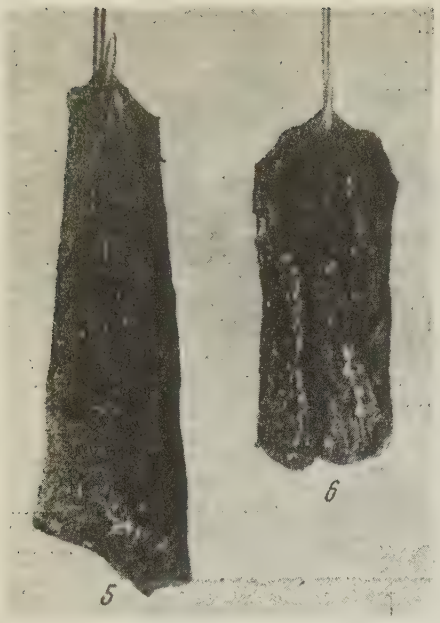


Fig. 2. Meat treated simultaneously with nystatin and chlortetracycline; separate mold colonies can be seen.

Group II, containing substances with a fungistatic effect at 30 to 250 μg/ml, includes three preparations: nystatin, vitamin $K_5$, and Arquad 2C.

Group III, containing substances with a fungistatic effect at 250 to 650 μg/ml, includes sorbic acid and preparation T-1.

Table 1 also reveals that there are three groups of preparations with different ranges of fungicidal action: Group I from 0.4 to 70 μg/ml, II from 30 to 500 μg/ml, and III from 500 to 2500 μg/ml. Group I contains K-25, Arquad C, Diocide, cetylpyridinium chloride, Katapin, and Kationat 2-B, II contains nystatin, vitamin $K_5$, and Arquad 2C, and III contains sorbic acid and preparation T-1.

In other words, the compositions of the groups of preparations according to their fungistatic or fungicidal action are exactly the same.

The antibiotics albofungin, griseofulvin and trichothecin in the form of culture fluids were found to be ineffective.

To test the effect of the antimold preparations on meat we made a selection from each group of preparations. From group I we took preparation K-25, which on degradation gives compounds such as bromine, rose oil, and acetic acid, which are commonly found in human food.

The quaternary ammonium compounds, despite their great effectiveness, were excluded from the tests on meat owing to their toxicity, but they can be used for the purpose of disinfection (Preobrazhenskii [1958]). From group II we selected only nystatin. Vitamin $K_5$ was found to be unsuitable for application on meat in view of its complete insolubility in water. Finally, from group III we isolated sorbic acid as being a harmless preparation and applicable for the preservation of many food products from mold.

Thus, three preparations—K-25, sorbic acid, and nystatin—were tested on meat.

Repeated experiments with K-25 on 400-500 g pieces of meat showed that this preparation, despite its effectiveness on pure cultures, did not check the growth of yeasts and molds on meat and, moreover, imparted a specific odor to the product. The preparation K-25 was excluded from subsequent experiments.

Sorbic acid in aqueous and aqueous-alcohol solutions, or in lactic acid, gave no positive results when the meat was treated. The inhibition of mold growth on meat for two days in these experiments was of no practical value, since the subsequent mold growth on the treated meat became equal to that of the control.

In the experiments on meat, nystatin was employed in combination with chlortetracycline, since we know that the former does not prevent bacterial spoilage. To treat the meat we prepared a solution containing a mixture of the two antibiotics. Water and 10% ethanol were used as solvents for the antibiotics. In the first experiments we used only 10% ethanol for better solution of the nystatin. Later we used an aqueous suspension of nystatin, which gave the same results as aqueous-alcohol solutions in the checking of meat molds. The experiments on the treatment of meat with a solution of nystatin and chlortetracycline were conducted in refrigerating chambers at air temperature 0 to +7 C and relative humidity 75 to 95%. For the tests we used slices of meat 5 × 8 cm and 0.5 - 1.0 cm thick, pieces weighing 400 - 500 g, and cuts weighing 5 - 6 kg. Six experiments were conducted altogether. For treatment of the meat with the solutions of antibiotics we employed the sprinkling method (as being the most simple and economic). The quantity of solution used on the two pieces weighing 400 - 500 g was 60 - 80 ml, and 1.5 - 2 liter was used on the two quarters.

To characterize the degree of mold growth on the meat we used the following terms: a) separate colonies—up to 10 mold colonies of diam 1-2 mm on a 400 $cm^2$ area of meat; b) weak growth—10 to 30 mold colonies of diam 1-5 mm on a 400 $cm^2$ area of meat; c) average growth—10-20% of the meat covered with separate colonies and small patches of mold; d) heavy growth—30-50% of meat covered with molds, individual colonies

TABLE 2

Degree of Development of Yeasts and Molds on Meat. Time of their Appearance (in days)

| No. of quarters | Concentration of chlortetracycline (CTC) and nystatin, μg/ml | Yeasts | | | Molds | | | |
|---|---|---|---|---|---|---|---|---|
| | | separate colonies | average growth on surface | heavy coating | separate colonies | weak growth | average growth | heavy growth |
| 2 | CTC, 100.0, and nystatin, 250.0, in 10% alcohol | 11 | — | — | 14 | 15—17 | — | — |
| 2 | CTC, 100.0, and nystatin, 250.0, in aqueous solution | 11 | — | — | 14 | 15—17 | — | — |
| 2 | CTC, 100.0 | 6 | 7 | 10 | 7 | 8 | 9 | 10 |
| 2 | Control | 5 | 6 | 7 | Removed from experiment because of bacterial spoilage | | | |

Note: The sign — denotes absence of indicated form of spoilage.

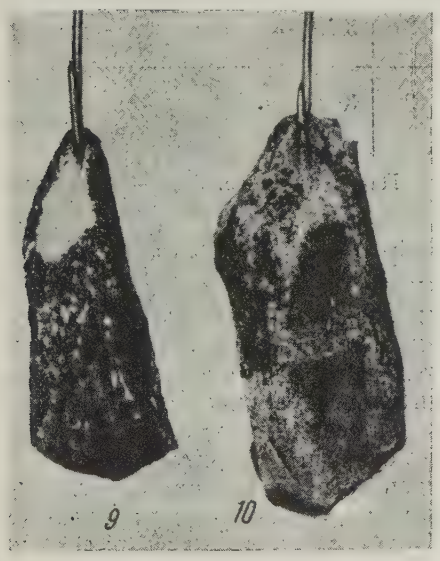


Fig. 3. Control meat not treated with antibiotics; heavy growth of molds and yeasts.

indistinguishable, the mold dense, hairy with hanging fragments. The same terms were also used to denote the growth of yeasts on the meat.

The results of experiments conducted on meat with the antibiotics nystatin and chlortetracycline showed that with a nystatin concentration of 250 ml/liter and chlortetracycline 100 ml/liter there was a suppression of mold growth for 5-7 days.

The rate of mold growth on the control meat and the meat treated with antibiotics was different. The mold growth on the treated meat began with separate colonies of diam 1-2 mm and by the end of the experiment consisted of separate colonies of diam 5-6 mm, whereas the control was 50-70% covered with large patches of mold. Meat treated with chlortetracycline alone was 60-80% covered with a continuous coating of mold. Figures 1-3 show the results of an experiment in which 10% alcohol was used as the solvent for the antibiotics. Identical results were obtained when aqueous and aqueous-alcohol solutions of antibiotics were used. Nystatin had a strong inhibitory effect on yeasts, too. On the treated meat the yeasts appeared 5 - 6 days later than in the control and consisted of separate colonies, the number and size of which were a little greater by the end of the period of meat storage.

It is of interest to note that the signs of bacterial spoilage of meat treated with chlortetracycline alone appeared 4 - 5 days earlier than on meat treated with chlortetracycline and nystatin. Hence, treatment of meat with nystatin in combination with chlortetracycline not only protected it from mold growth, but also from bacterial spoilage, and the total time of preservation of the meat was 9 - 10 days more than that of the control.

The positive results of the laboratory experiments on slices, pieces and cuts of chilled meat enabled us to advance to experiments on quarters of beef in semiplant conditions.

The method of preparing the solutions, the treatment of the meat and inspection of it were the same as in the laboratory experiments. The meat storage conditions were stricter — the mean temperature was + 3 C and the relative air humidity 90%.

The control quarters were removed from the experiment before the onset of mold growth, since the signs of bacterial spoilage found after seven days did not allow us to preserve the meat any longer.

The growth of molds and yeasts on meat treated with chlortetracycline alone and a mixture of chlortetracycline and nystatin is illustrated in Table 2.

As Table 2 shows, yeasts appeared on the chlortetracycline-treated meat and on the control meat on the 5th to 6th day and by the 7th-10th day heavily covered it. At the same time, no yeasts were detected on the meat treated with the two antibiotics until the 11th day, when separate colonies appeared at scattered points, and a slight increase in their size and number during the rest of the experiment was noted.

Molds appeared on the chlortetracycline-treated meat on the 7th day and during the three following days spread rapidly over the whole surface of the meat, on the brisket, near the pubic bone, on the flank, and especially on the neck area. On meat treated with the two antibiotics, the molds appeared on the 14th day of storage in the form of separate colonies of diam 1-1.5 mm and by the end of the experiment the growth was still slight.

Meat treated with the two antibiotics was easily distinguishable in external appearance as well. While the control meat by the 7th day had become much darker, to say nothing of the mat film of yeasts, the meat treated with nystatin and chlortetracycline still retained its bright red color by the 17th day. On it we observed only separate colonies of yeasts and molds. Meat treated with chlortetracycline alone lost its market appearance on the 8th-9th day, this being mainly due to the heavy growth of yeasts and molds on its surface.

Among other observations we may note that nystatin mainly prevented the growth of molds of the genus Mucor. On control meat and meat treated with chlortetracycline alone, molds of the genus Mucor appeared three to four days earlier than fungi of the genus Penicillium and during the following three days spread rapidly over most of the surface. When the meat was treated with chlortetracycline and nystatin, only fungi of the genus Penicillium developed. The rate of their development was weak: within 5-7 days the diameter of the colonies increased by 5-8 mm.

## SUMMARY

1. A study was made of the fungistatic and fungicidal effect of 16 antimold preparations on pure cultures of molds.

2. It was found that nystatin (250 mg/liter) in combination with chlortetracycline (100 mg/liter) retarded mold growth on meat by 5-7 days as compared with the control and considerably slowed down the further spread of initiated mold growth (at 0-2 C, 3-5 C, 5-7 C and relative humidity 75 to 95%).

On meat treated with these two antibiotics, yeasts appeared 5-6 days later than in control meat or on meat treated with chlortetracycline alone, and consisted of separate colonies which did not increase greatly in size or number.

3. Aqueous solutions of nystatin gave the same results on meat as 10% aqueous-alcohol solutions of this antibiotic.

4. Treatment of meat with nystatin and chlortetracycline together increased the storage life of meat by 9-10 days in comparison with the control.

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# ELECTRON MICROSCOPY OF ONION MOSAIC VIRUSES

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Two viral diseases have been found in onions. One of these—the mosaic disease described by Ryzhkov and Vovk (1937)—starts as pale yellow or white stripes on the leaves, followed by the onset of general chlorosis of their leaves and their drooping. The inactivation temperature of the virus which causes this disease is 60 C (Vovk, 1956).

Ryzhkov (1946) describes still another viral disease of onions—dwarf yellows. This disease starts with the appearance of narrow yellow stripes which increase in size. The leaves curl downward, and the plant is stunted in development. According to the author, this virus can affect garlic, some species of lilies, and narcissus. The inactivation of the virus which causes the latter disease occurs at 75-80 C.

A similar disease of onions has also been described in Klinkovskii's work.

We know of only two works devoted to the electron microscopy of viruses causing disease in onions (Smirnova, 1953 and Vovk, 1956). The authors of these works point out that they observed spherical particles with a diameter of 200-250 mμ in the juice of onions affected by mosaic. Smirnova believes that these particles are included in the composition of the X-bodies which can be observed in the cells of affected onions. Both X-bodies and the spherical particles observed with the electron microscope are absent from the cells of healthy plants. In addition to this, in several cases she observed long (filamentous) rods in the juice of diseased onions which were similar to the viral particles of the X-virus of potatoes or of lily mosaic. Smirnova did not regard these fine rods as viral particles. In one case, she saw similar rods in the juice of outwardly healthy onions.

Using the electron microscope, we investigated both diseased and healthy green onions grown in greenhouses in February and March.

In diseased plants, long, frequently interrupted, narrow, pale green stripes were observed along the length of the leaves. Later, leaves appeared which were chlorotic, elongated, thinner than in normal plants, and had low turgor; the bulbs of such plants were more frequently elongated. When the symptoms of the disease are compared, it is seen that it is similar to the mosaic disease described by Ryzhkov and Vovk. In our case, there were up to 10% of such plants in the greenhouse.

Preparations for electron microscopy were made from small pieces of leaves (approximately 0.5 $cm^2$) ground with sand in a mortar. We added 5-10 ml of distilled water to the ground mass and mixed it thoroughly. A small drop of homogeneous suspension was placed on the film and was examined under the microscope after spraying with chromium.

The picture observed when an aqueous solution obtained from a healthy plant was examined is presented in Fig. 1. The stretched-out, formless clumps of various sizes represent cellular elements which have been disrupted during grindings.

In Figs. 2, 3 and 4, which were obtained at the same magnification using preparations of diseased plants having the symptoms indicated above, three types of particles can be seen: 1) filamentous, wavy, rather thin (12 mμ) particles (Fig. 2); 2) filamentous, wavy, thicker (25 mμ) and considerably longer than particles of the

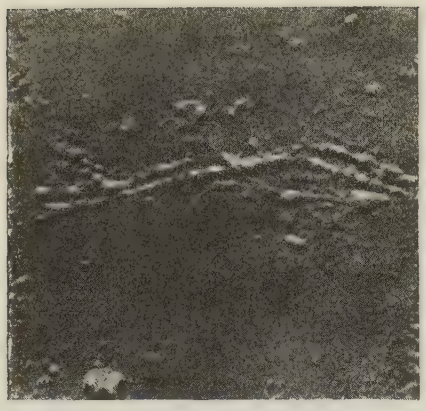

Fig. 1. Aqueous suspension of tissues from healthy onion under the electron microscope. Magnification 20,000 ×.

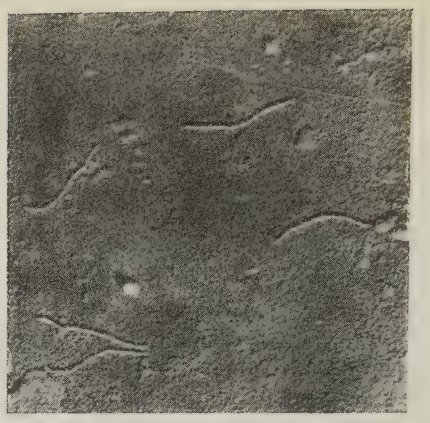

Fig. 2

Fig. 3

Fig. 4

Fig. 2-4. Aqueous mixture of tissues from onion affected by mosaic disease, in the electron microscope. Magnification 20 000 X.

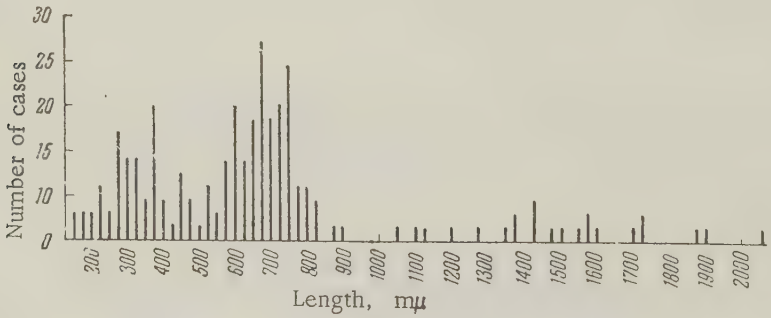


Fig. 5. Length of filamentous particles observed in electron microscope.

first type (Fig. 3); one particle of the first type is seen; 3) small clumps approaching the spherical in shape, similar to the particles mentioned in the works of Smirnova and of Vovk (Fig. 4); particles of the first type are also seen.

Filamentous or rod-shaped particles found during electron microscopy of plants affected by viruses are considered to be viruses by the majority of plant pathologists. For some viruses, this has been proved experimentally.

Different viruses have particles of various lengths and shapes—spherical as well as filamentous and rod-shaped. The shape and dimensions of viral particles are one of the characteristics of viruses according to which their systematic position is determined.

We are therefore also inclined to regard the filamentous particles found in diseased plants as virus particles. Neither do we have any reason to deny the viral nature of the spherical particles described in the works of Smirnova and Vovk and observed in Fig. 4. We infected the young leaflets of 20 bulbs of seed onions with the juice from the leaves of diseased plants. The inoculations were made by needle pricks through drops of the infectious liquid placed on the leaves of the plants.

There were no outward symptoms of the disease in 35 days after inoculation. When preparations of the plants were examined in the electron microscope five days following inoculation, no differences from healthy plants were found. When the same plants were examined 35 days after inoculation, wavy, filamentous particles such as represented in Fig. 2 were found, singly or in pairs, but not in every field of the microscope.

We measured 181 filamentous particles observed in the aqueous extracts of diseased plants.

The results of the measurements are presented in Fig. 5.

It is seen from Fig. 5 that the most frequently encountered length of the filamentous particles was 675 mμ, and that half of all of the particles measured were from 600 to 800 mμ in length.

The group of filamentous particles with a length greater than 1000 mμ draws attention to itself. Twenty-three of these were noted, half of them having a length of from 1400 to 1600 mμ. In Fig. 3, they differ from the shorter particles not only by considerably greater length, but thickness as well.

The spherical particles mentioned in the works of Smirnova and of Vovk were also noted in our electron photomicrographs (Fig. 4). Measurements of 23 particles showed that their dimensions fluctuate from 150 to 250 mμ, i.e., they are the same as in the works of the above-mentioned authors. Smirnova also noted the presence of filamentous particles in mosaic-affected onions.

## SUMMARY

Electron microscopy of onion plants which had been infected with mosaic disease both naturally and artificially showed the presence of three different morphologically distinct viruses in the leaf tissues: filamentous —675 mμ, filamentous—1500 mμ, and spherical—200 mμ. So far, it has not been possible to make virological differentiations and to obtain symptoms for each of these viruses.

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**All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal.** ***Some or all of this periodical literature may well be available in English translation.*** **A complete list of the cover-to-cover English translations appears at the back of this issue.**

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# THE MOST FAVORABLE AMOUNT OF "DRY NUTRIENT AGAR" IN MEDIA FOR THE CULTIVATION OF HETEROTROPHIC MARINE MICROORGANISMS

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In order to isolate heterotrophic marine microorganisms, ready-made "dry nutrient agar" consisting of tryptic hydrolyzate of fish meal (60%) and agar (40%) is frequently used. Fifty g of dry nutrient agar is added to 1 liter of sea water.

As is well known, a very small percent of the total number of microorganisms which can be counted by the direct count method grows on peptone media (Show and Fred, 1926; Razumov, 1953; Butkevich, 1932; Kriss, 1945, and others).

The purpose of the present work was to find media which would be more favorable for the growth of microorganisms than dry nutrient agar, ensuring the formation of a large number of colonies while retaining the diversity of their forms.

TABLE 1

Composition of Media Used for Culturing Heterotrophic Marine Microorganisms

| Components of medium | Media contained (per 1000 ml of fresh water) | | | | | | |
|---|---|---|---|---|---|---|---|
| | № 1 | № 2 | № 3 | № 4 | № 5 | № 6 | № 7 |
| Dry nutrient agar, g | 50.0 | 37.5 | 25.0 | 12.5 | 5.0 | 2.5 | 0 |
| Same, % of its content in ordinary meat-peptone | 100 | 75 | 50 | 25 | 10 | 5 | 0 |
| Agar, g | 0 | 3.75 | 7.5 | 11.25 | 13.5 | 14.25 | 15 |

It is also known that a larger number of microorganism colonies sometimes develops on "starvation" media (agar in water) than on ordinary peptone media. Kriss and Rukina (1952) note that colonies of microorganisms grow on "starvation" agar (agar with sea water) even when their growth is not observed on ordinary meat- or fish-peptone media. Razumov (1953) also cultured heterotrophic bacteria on "starvation" media, and according to his data, from 1/10 to 1/60 of the microflora which can be counted by direct microscopy grew on such media.

He counted the colonies which grew after several weeks; the colonies were very small in size. Other authors have also mentioned increased numbers of colonies when media are diluted. However, we found no indications in the literature of what degree of dilution of the medium is the most favorable for culturing heterotrophic marine bacteria and whether their qualitative composition changes when ordinary dry nutrient agar is diluted.

TABLE 2

Comparative Data on the Number of Colonies of Heterotrophic Bacteria Growing Out when Sea Water from Sevastopol Bay Was Inoculated on Dry Nutrient Agar.

| Date, 1958 | Average figures* | Med. No. 1 (contr.) | | Medium No. 2 | | Medium No. 3 | | Medium No. 4 | | Medium No.5 | | Medium No. 6 | | Medium No. 7 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | number of colonies | % | number of colonies | % of medium no. 1 | number of colonies | % of medium no. 1 | no. of colonies | % of medium no. 1 | no. of colonies | % of med. no. 1 | number of colonies | % of medium no. 1 | number of colonies | % of medium no. 1 |
| Oct. 31 | $x_1$ | 106 | 100 | 330 | 311 | 445 | 420 | 460 | 433 | 670 | 632 | 376 | 354 | 91 | 86 |
| Nov. 11 | $x_2$ | 113 | 100 | 68.5 | 60.5 | 188 | 166 | 120 | 106 | 162 | 143 | 99 | 87.5 | 79.5 | 70.5 |
| Nov. 23 | $x_3$ | 72 | 100 | 145 | 202 | 202 | 280 | 160 | 223 | 125 | 174 | 99.5 | 132.5 | 13.3 | 18.5 |
| Dec. 8 | $x_4$ | 119 | 100 | 350 | 294 | 790 | 662 | 840 | 705 | 485 | 407 | 125 | 105 | 53.5 | 45 |
| Dec. 12 | $x_5$ | 130 | 100 | 550 | 423 | 450 | 346 | 575 | 442 | 490 | 377 | 78.5 | 60.4 | 32.4 | 24.9 |
| | $x$ | | 100 | | 258.0 | | 375 | | 382 | | 347 | | 148 | | 49 |

*$\overline{x_1 - x_5}$ – Ratios of the averages of individual media to those of the control, expressed in percent; $x$ – average for average figures $x_1 - x_5$ and averages of diversity of forms.

In order to resolve this problem, we used a series of dilutions of dry nutrient agar from its normal composition to "starvation" medium, while adding various amounts of agar (Table 1).

In order to keep the same density of the media, agar was added in the amount of approximately 1.5%. The media were poured into Petri dishes and were dried slightly at 30-40 C.

Inoculations were made with 0.04 ml of fresh water from Sevastopol Bay. The plates were kept at room temperature (18-20 C).

The number of colonies growing was always counted after 2 and 5 days, and in certain cases, the diversity of the colony forms of the microorganism was also recorded.

The experiment was set up in three simultaneous repetitions with each of the test media, and was repeated five times during the November–December period in 1958.**

In the experimental series, the arithmetic mean of three repetitions was calculated for each individual medium. The arithmetic means of the control (ordinary dry nutrient agar – medium No. 1) were calculated in the same manner and used as 100% each time. The ratios of the average figures for each medium to the averages of the control ($x_1 - x_5$) were totaled, and the average ($x$) characterizing the individual media was calculated from them. The average figures ($x$) were compared among themselves*** (Table 2).

A comparison of the average figures ($x$) for each individual medium showed that the largest number of colonies grew on media Nos. 2, 3, 4, and 5 (Table 1), exceeding the number of colonies on ordinary dry nutrient agar (medium No. 1) by 2.5 - 3.8 times.

The colonies on media Nos. 2, 3, and 4 were approximately of the same size as the colonies on medium No. 1. On medium 5, the colonies were 2-3 times smaller, but were more uniform in size. The greatest diversity of colony forms was observed on media Nos. 4 and 5. (7.5 and 8.4, respectively).

Owing to the lower content of dry nutrient agar, diluted media are more transparent as compared with ordinary peptone ones, which greatly facilitates counting of colonies and their morphological differentiation.

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**Preparator E. G. Vorontsova participated in making media and in counting the colonies.

***The method employed can be recommended for the treatment of mass microbiological materials.

TABLE 3

Quantitative Count of Heterotrophic Bacteria on Media Nos. 1, 5 and 7 (inoculum – 0.04 ml of sea water)

| Date, 1959 | Average figures* | Medium No. 1 (control) | | Medium No. 5 | | Medium No. 7 | |
|---|---|---|---|---|---|---|---|
| | | number of colonies | % | number of colonies | % of medium No. 1 | number of colonies | % of medium No. 1 |
| May 5 | $x_1$ | 161.5 | 100 | | | 248 | 153 |
| May 13 | $x_2$ | 73.4 | 100 | | | 70.2 | 96 |
| May 21 | $x_3$ | 560 | 100 | 857 | 153 | 1600 | 286 |
| June 1 | $x_4$ | 329 | 100 | 856 | 260 | 314 | 95.5 |
| June 9 | $x_5$ | 935 | 100 | 1205 | 129 | 151.5 | 16.2 |
| June 16 | $x_6$ | 282 | 100 | 551 | 195 | 160 | 56.5 |
| June 22 | $x_7$ | 343 | 100 | 394 | 115 | 21 | 6.12 |
| July 17 | $x_8$ | 272 | 100 | 1180 | 435 | 715 | 263 |
| Sept. 9 | $x_9$ | 340 | 100 | 547 | 161 | 932 | 274 |
| Sept. 23 | $x_{10}$ | 372 | 100 | 386 | 103.5 | 292 | 59.8 |
| Oct. 7 | $x_{11}$ | 353 | 100 | 328 | 93 | 126 | 35.7 |
| Oct. 15 | $x_{12}$ | 683 | 100 | 922 | 135 | 612 | 89.8 |
| Oct. 23 | $x_{13}$ | 332 | 100 | 590 | 177.5 | 320 | 96.2 |
| x ±M | | | 100 | | 178 ± 29.5 | | 117.5 ± 30.5 |

*$x_1 - x_{13}$ – ratio of averages for individual media to the averages of the control, expressed in percent; x – arithmetic mean of average figures $x_1 - x_{13}$; m – deviation from arithmetic mean x.

TABLE 4

Number of Microorganisms in 0.04 ml of Sea Water when Counted by the Direct Count Method and on Agar Dishes

| On agar plates | | | | By the direct count method on filters |
|---|---|---|---|---|
| starvation medium number 7 | diversity of forms | DNA (medium No. 1) | diversity of forms | |
| 238 ± 17.1 | 4.25 | 47 ± 3.5 | 5.4 | 10400 |

The number is the average value; ± are the deviations from the average.

We selected medium No. 5 for the work, on which the colonies of microorganisms were relatively similar in size and showed the greatest diversity in form. Medium No. 1 and "starvation" agar (medium No. 7) served as the controls.

This series of experiments was conducted in quadruplicate. The treatment of the numberical material was the same as in the first series of experiments. In addition, series consisting of the ratios of the arithmetic means for the media to those for the control, expressed in percent ($x_1 - x_{13}$), were treated statistically in order to obtain reliable averages (x) and deviations of these averages (m) (Zdorik, 1952; Fisher, 1952) (Table 3).

A comparison of the average figures (x) for each medium shows that approximately twice as many colonies grew on medium No. 5 as on medium No. 1. The growth rate of the colonies was the same on both media. An average of 1.2 times more colonies of microorganisms grew on "starvation" medium No. 7 than on dry nutrient agar, but their number changed very greatly during the course of the year, and the average is very relative. Five days of cultivation is evidently insufficient in the given case.

It is interesting to note that on diluted media (Nos. 2, 3, 4, and 5) the number of colonies which grew in

five days was usually from 50 to 200, and sometimes as high as 1000% of their number counted on the second day. Moreover, even after two days, 1.5 - 2 times more of them usually grew than on medium No. 1. At the same time, on medium No. 1, an increase in colonies was observed on the fifth day (from 10 to 50%, and only rarely as high as 200%). On media Nos. 3, 4, and 5, a richer variety of brightly colored colonies was always observed than on ordinary dry nutrient agar, and various agar-liquefying forms, which were usually rare on medium No. 1, always developed.

It should also be noted that on medium No. 5 the colonies of microorganisms were clearly demarcated from one another, and almost never coalesced, even upon long standing.

Diluted media Nos. 2, 3, 4, 5 and 6 were not more favorable than medium No. 1 for maintaining germinating bacterial cultures isolated from various seas and oceans. Attempts to revive cultures which exhibited constantly poorer growth from transfer to transfer did not meet with success.

A comparison was made of the number of colonies of heterotrophic marine microorganisms growing on "starvation" medium and the data of direct counts.

The following experiment was set up: 32 dishes of "starvation" medium and 10 of ordinary dry nutrient agar (medium No. 1) were each inoculated with 0.04 ml of fresh sea water from Sevastopol Bay.

Twenty ml amounts of three samples of the same water were filtered through membrane filters for counts of the total number of microorganisms by Razumov's direct count method (1931). After five days of exposure, the number of colonies and diversity of forms was recorded for the 10 plates of dry nutrient agar, and after 30 days the same was done with the "starvation" medium (31 dishes).

The results obtained were reduced to ranges and were treated statistically in order to determine reliable averages and deviations from the averages (Table 4).

When average figures are compared, it can be noted that five times more colonies of microorganisms grew on "starvation" medium than on ordinary dry nutrient agar with somewhat less diversity of forms than on the latter. The number of microorganisms determined by direct counts was found to be 24 and 124 times greater than the number of colonies growing on "starvation" medium (we conditionally assumed that one colony grows from one bacterial cell), if the extreme values of numbers of colonies in the series are taken. On the average, however, 1/52nd of the number of microorganisms recorded by direct counts grew on "starvation" medium. Razumov (1953) found that the ratio is from 1/10 to 1/60 for fresh-water heterotrophic bacteria. However, the colonies of marine heterotrophic bacteria growing on "starvation" medium are usually very minute (millimeters and fractions of millimeters), they are less diverse in form than on dry nutrient agar, and their growth time is longer (to 30 days). All of these reasons do not permit the wide use of "starvation" medium in marine microbiology, especially under expeditionary conditions. On the basis of the results obtained (Table 2), it can be considered as established that it is not expedient to dilute dry nutrient agar more than 10 times.

Media diluted 2, 4, and 10 times (Nos. 3, 4 and 5) were the most favorable for the growth of marine heterotrophs. Medium No. 5 (diluted 10 times) made it possible to detect an average of 1.78 times more heterotrophic marine microorganisms than dry nutrient agar, which is usually employed for this purpose. It can therefore be recommended for studies of heterotrophic marine microorganisms; this is also true of media Nos. 3 and 4, which permit the most complete detection of numbers and species composition of these microorganisms during the course of the entire year.

## SUMMARY

1. The testing of media obtained by means of the simple dilution of dry nutrient agar showed that the most favorable medium for the growth of heterotrophic marine microorganisms is dry nutrient agar diluted 10 times (medium No. 5). This medium not only gives an approximately twofold increase in the number of colonies as compared with ordinary dry nutrient agar and a greater diversity of colony forms of the microorganisms, but on it and on media diluted two and four times (Nos. 3 and 4), the ability of the microorganisms to liquefy agar and to produce pigment is not lost.

Media Nos. 3, 4 and 5 can be recommended for studying heterotrophic marine microorganisms.

2. Five times as many colonies of marine microorganisms grow on "starvation" medium (agar in sea water) as on dry nutrient agar.

On the average, this figure is 1/52nd part of the number of microorganisms which can be detected by the direct count method, but due to the long period of incubation (30 days), the very small size of the colonies (millimeters or fractions of millimeters), and the relatively small variety of forms, this medium can not be widely used for the isolation and study of heterotrophic marine microorganisms.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

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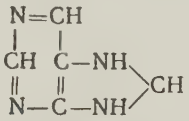
# PURINE BASES AND THEIR ROLE IN BACTERIAL METABOLISM

E. L. Ruban

Translated from Mikrobiologiya, Vol. 30, No. 1, pp. 173-180,
pp. 173-180, January-February, 1961

Purine bases, i.e., purine derivatives, are widely distributed among the metabolic products of animals and plants. Since they are included in the composition of nucleic acids, purine bases participate in the processes of conversion of nuclear material (purine bases → nucleosides → nucleotides → nucleic acids).

Individual purine bases are links in the conversion chain of purine.



Purine is two urea residues joined to a three-carbon chain. Pure purine is not encountered in nature, and was obtained artificially by the reduction of uric acid (Blagoveschenskii, 1958).

The derivatives of purine are adenine, guanine, hypoxanthine, xanthine, uric acid, allantoin, and allantoic acid.

All of the derivatives of purine have been found in plant and animal tissues. Aside from nucleic acids, bacterial cells contain nucleotides, nucleosides, and free bases (Mesrobeanu, 1936; cited by Stephenson, 1951).

In bacteria purine nitrogen constitutes around 7.8 to 9.4% of total nitrogen. The total amount of purine nitrogen constitutes from 0.20 to 0.30% of the weight of wet bacteria. This amount is exceeded only by the amount of purine nitrogen in thyroid gland tissue.

Work on the distribution of purine bases has shown that adenine is predominant among bacterial purines; bacterial nucleotides and nucleosides contain xanthine and hypoxanthine in the least amounts (Stephenson, 1951). The metabolism of purine bases in bacteria essentially amounts to three points: 1) the ability or inability to synthesize the individual purine bases, and in connection with this the requirement for purines or its absence; 2) the utilization of external purines as nutrient sources; 3) the incorporation of existent purines into the composition of nucleic acids.

## Requirement by Microorganisms for Purine Bases

Many lactic acid bacteria require adenine, guanine, hypoxanthine, and xanthine for growth; they apparently cannot synthesize these substances from the components of the nutrient medium. All four purine bases are interchangeable.

It has been established that lactic acid bacteria require $B_{12}$; however, this requirement for the vitamin can be eliminated by the addition of hypoxanthine, adenine, guanine, or cytosine to the medium (Shive, 1951; Werkman and Wilson, 1951).

It is possible that purine bases participate directly or indirectly in the synthesis of vitamin $B_{12}$ (Aaronson, 1958).

When culturing Lactobacillus casei and Streptococcus faecalis, the requirement of these microorganisms for folic acid can be eliminated by adding thymine and purine bases to the medium. The requirement of Acetobacter suboxidans for para-aminobenzoic acid is ten times higher when it is cultured in medium free of purine bases than in their presence. This has also been observed in Lactobacillus arabinosus (Werkman and Wilson, 1951). Paraaminobenzoic acid possibly catalyzes the synthesis of purine bases, and in those cases when these substances are present as such in the medium, the requirement for paraaminobenzoic acid decreases.

Thus, in the absence of the vitamins which catalyze the synthesis of purine bases, they can be replaced by existing purine bases. The easy interchangeability of the individual purines suggests that, in the bacterial cell, they are evidently constantly being transformed from one to the other.

## Breakdown of Purine Bases (Aerobic)

Early investigations of the breakdown of purines were frequently carried out with impure cultures, and are therefore only of historic interest.

In 1905, Ulpiani and Cigolam (cited by Franke and Hahn, 1955) isolated a Gram-positive aerobic coccus from chicken excrements which decomposed uric acid and guanine. $NH_4$, $CO_2$ (with guanine), and guanidine were recorded as the reaction products.

Liebert (1909) investigated a number of organisms of the genus Pseudomonas—P. aeruginosa, P. fluorescens, P. eisenbergii, P. calcoacetica—and found that the decomposition of guanine proceeds through allantoin, uric acid, and oxalic acid. Micrococcus forms $NH_4$, $H_2$, and $CO_2$ as the end products of purine decomposition. Certain representatives of this genus are distinguished by the production of side products; thus, in Micrococcus lactis, decomposition proceeds according to the following pathway:

hypoxanthine → xanthine → uric acid → $NH_3$, $H_2$, $CO_2$ + propionic acid.

Instead of propionic acid, Micrococcus aerogenes forms lactic acid and a small amount of pyrimidines (thymine and uracil).

Di Fonso's investigations (1952) with various spedies of Mycobacterium (M. smegmatis, M. stereoris, M. butyricum, M. phlei, M. tuberculosis) showed that the breakdown of purines in these microorganisms proceeds through uric acid, allantoin, allantoic acid, urea and ammonia.

Krebs and Eggleston (1939) found that Corynebacterium ureafaciens splits off urea from a whole series of compounds—hypoxanthine, uric acid, allantoin, allantoic acid—by means of adaptive enzymes. Franke and Hahn (1955) studied P. aeruginosa, which decomposes purine with great intensity. It was established that some strains of P. aeruginosa break down purines according to the following pathway:

uric acid → allantoin → allantoic acid → urea + glyoxalic acid

urea → $CO_2$ + $NH_3$; glyoxalic acid → oxalic acid.

It was possible to stop the reaction at the intermediate products stage by using high concentrations of the initial compounds.

Cambell (1954, 1955), who was studying the activity of cell extracts from two strains of Pseudomonas, established that the breakdown of uric acid proceeds by the following pathway:

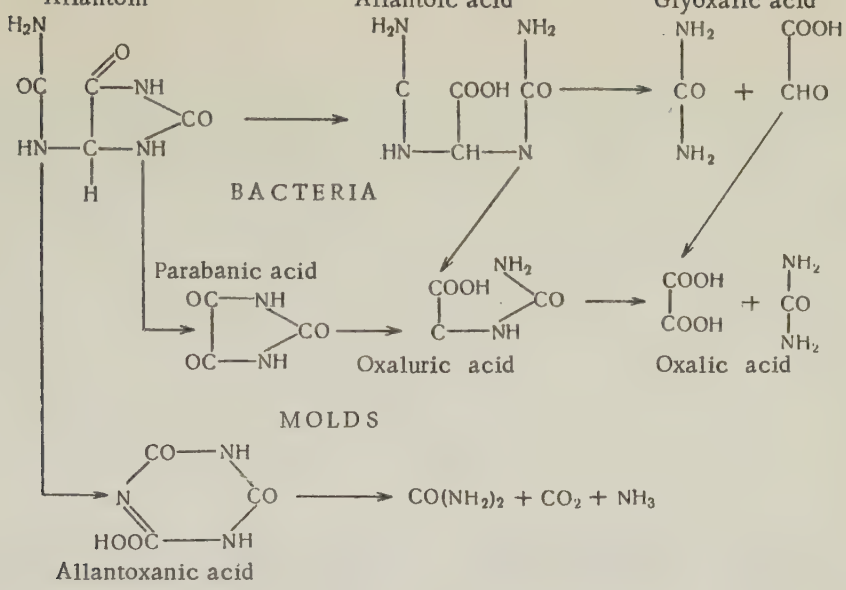
uric acid → allantoin + $CO_2$ → allantoic acid → glyoxalic acid + urea → formic acid → $CO_2$ + $H_2O$.

One of the strains investigated oxidized glyoxalic acid to oxalic acid and hydrolyzed urea to ammonia and carbon dioxide; the other strain oxidized glyoxalic acid to carbon dioxide and water, but did not hydrolyze urea.

The works of Young and Hawkins (1944) show that Escherichia coli breaks down allantoin according to the following scheme:

allantoin → allantoic acid → urea + glyoxalic acid → ammonia + $CO_2$ + oxalic acid.

The possible cases of allantoin decomposition by bacteria and molds can be expressed by the following scheme 1 (Krebs and Eggleston, 1939):

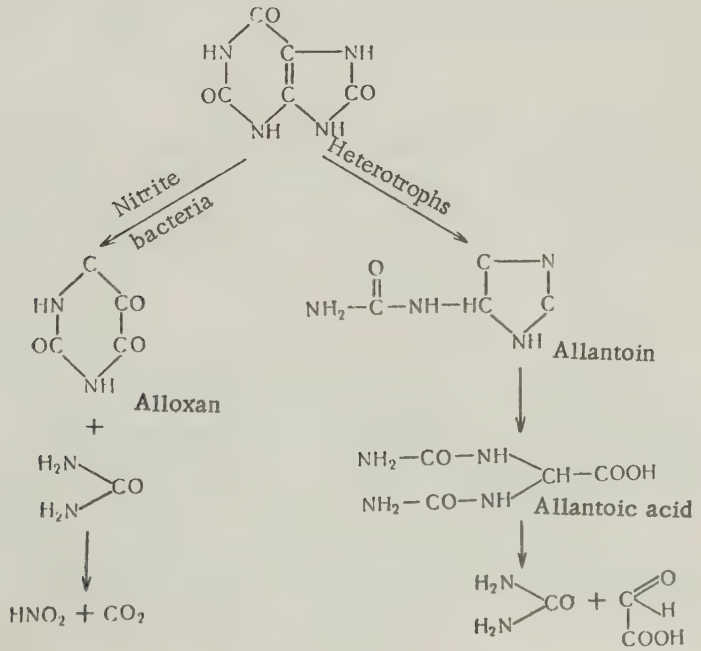



Not all aerobic microorganisms are capable of decomposing purines to ammonia and carbon dioxide; thus, for example, M. butyricum stops at the urea stage, while M. phlei does not go beyond the uric acid stage. A microorganism isolated by Dubos and Miller and named Corynebacterium ureafaciens has a high oxidative capacity and oxidizes amino acids, purine bases, uric acid, and all compounds containing the N–C–N group; the end product of the oxidation is urea (Krebs and Eggleston, 1939), which is formed in the amount of 100% of theoretical.

Nitrifying bacteria (Nitrosomonas europaea) can carry out the process of nitrification at the expense of the amino groups of guanine, allantoin, and uric acid. However, the question of whether this involves the direct deamination of the purine derivatives or whether they are first broken down with the production of urea and ammonia has not yet been clarified (Ruban, 1958).

In cultures of nitrite bacteria, uric acid is evidently not broken down through allantoin, glyoxalic acid, and urea, as is the case in heterotrophic bacteria, but involves the formation of another compound.

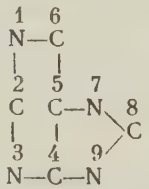
It is known that, in the presence of nitric acid, uric acid can be oxidized with the production of alloxan. Since large amounts of $NO_2$ ions and some amount of $NO_3$ ions are usually present in cultures of nitrifying microorganisms, the possibility of alloxan production seems quite probable. The medium itself in which the nitrite bacteria grow can influence the direction of the oxidation of uric acid (Ruban, 1960). Thus, we can envision two pathways of conversion for uric acid (scheme 2):



## Anaerobic Breakdown of Purine Bases

The pathways of anaerobic breakdown of purines differ from aerobic breakdown. Liebert (1909) observed the breakdown of uric acid with the formation of ammonia, acetic acid, and $CO_2$ by Clostridium acidi-urici. Clostridium cylindrosporus gives a small amount of glycine in addition (Franke and Hahn, 1956).

By using the labeled atoms method, it was possible to demonstrate that the breakdown of the purine molecule by the representatives of the genus Clostridium occurs in the following manner:



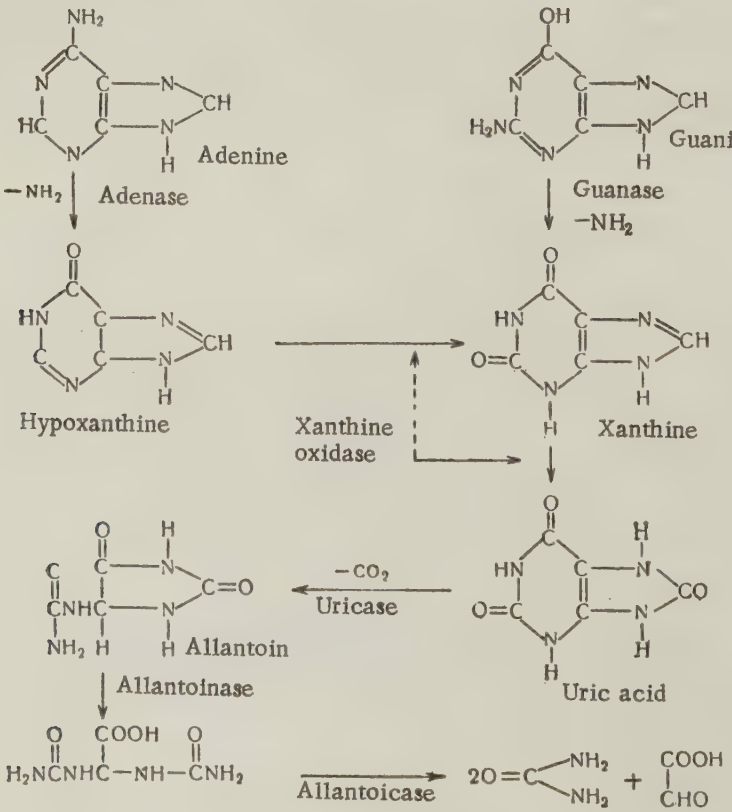
$CO_2$ is formed from atoms 2, 4, and 8, while methyl-labeled acetic acid is formed from atoms 2 and 8. Carboxyl-labeled acetic acid is formed from atom 5 (Franke and Hahn, 1955). About one-fifth of the acetic acid is formed synthetically from $CO_2$.

Barker (1943) showed that Streptococcus allantoicus breaks down allantoin anaerobically with the formation of ammonia, urea, and formic, acetic, lactic, and oxamic acids.

Young and Hawkins (1944) investigated the decomposition of allantoin by intestinal bacteria including the strict anaerobes, Clostridium putrificus and Lactobacillus bifidus. No decomposition of allantoin occurred in cultures of strict anaerobes.

Thus, the decomposition pathways of purines by various groups of microorganisms coincide in the first stages of decomposition, and divergence is observed only in the end product stages.

The enzymes participating in the conversion of purine bases are widely distributed among animals, plants, and microorgansims. They are quite specific, and each of them is responsible for a particular step in the decomposition. The participation of the different enzymes and the sequence of their action are represented in scheme 3 (Clifton, 1957):

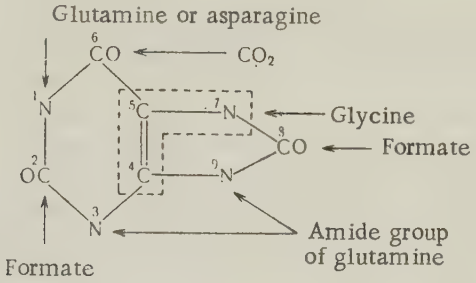


Investigations of purine deaminases in cultures of Bacterium breslau (Zakharova, 1958) showed a connection between the process of deamination and the oxidation of other compounds in the cell, and the stimulatory effect of deamination on the metabolic processes of the cultures.

## Synthesis of Purines and Nucleic Acids

The synthesis of purine bases occurs at the expense of amino acids, free ammonium ions, carbon dioxide, and formic acid.

The scheme of the incorporation of different compounds into the purine ring of uric acid, which was obtained on the basis of investigations of both microorganisms and higher animals (Chargaff and Davidson, 1957) with the application of the labeled atoms method, has the following appearance (scheme 4):




Investigations to determine precisely which substances serve as the initial material for the synthesis of the purines of nucleic acids showed that these substances are quite varied and that the pathways for their incorporation are similar in both higher animals and in microorganisms. Abrams, Hammersten, and Shemin (1948) showed that glycine is the material for purine synthesis in the yeast Torula utilis. Lactobacillus casei grows on guanine, hypoxathine, or xanthine as the purine source, but cannot utilize 2,6-diaminopurine as the precursor for purines (Elion, Vander Werff, and Hitching, 1953).

E. coli utilizes adenine, guanine, diaminopurine, adenylic acid, adenosine, guanosine, inosine, and diaminopurine-riboside for the synthesis of RNA and DNA, but the extent of utilization is different for each substrate (Balis and Lark, 1954).

Guanine, guanylic acid, and guanosine are used by E. coli as a source of bacterial guanine, but are converted to adenine in insignificant amounts (Bolton and Reynard, 1954).

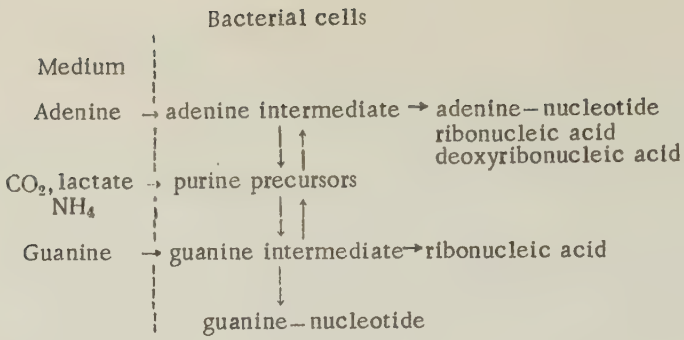
E. coli utilizes external purine compounds similar to those incorporated into its nucleic acids, and only after this does it begin to synthesize them de novo. The conversions of these compounds are specific; thus, adenine is converted to guanine and cytosine, purines are not converted to pyrimidines, and pyrimidines are not converted to purines (Bolton and Reynard, 1954; Bolton and Mandel, 1957).

Microorganisms (Torula utilis, Aerobacter aerogenes, Lactobacillus leishmanii) can utilize existing purines for incorporation into their nucleic acids (Chargaff and Davidson, 1957). In studying purine synthesis in A. aerogenes cultures (Sutton and Werkman, 1953), it was established that glycine is incorporated into the 4, 5, and 7 positions of adenine and guanine, $CO_2$ is incorporated into the 6 position and the 2 + 8 position through reduction to formaldehyde. Nitrogen atoms 1, 3, and 9 can be incorporated from ammonium sulfate of the medium.

L. casei incorporates guanylic and adenylic acids into nucleic acid (Balis, et al., 1952, 1953, 1954).

When existing purines are utilized for the synthesis of nucleic acids, no direct incorporation of these compounds into the nucleic acid molecule apparently occurs. They must be altered in some way, passing through the intermediate product stage prior to incorporation into nucleic acid (Koch, Putnam, and Evans, 1952). It was established that, when E. coli is grown on medium containing $C^{14}O_2$ and carbon-labeled purines, the carbon of $C^{14}O_2$ is incorporated into the 6 position of the purine molecule in both nucleic acids. Labeled adenine and guanine are also incorporated into nucleic acids. When adenine or guanine is present in the medium separately, the conversion of guanine to adenine and the reverse occurs (Bolton and Reynard, 1954).

The suggestion has been made that it is not external purines, but rather intermediate products similar to them, which are the precursors of nucleic acids (Koch, Putnam, and Evans, 1952):




Investigations of the metabolism of azapurines (Smith and Matthews, 1957) showed that these compounds are incorporated into the nucleic acids of E. coli. 8-Aza-guanine participates in the synthesis of the nucleic acids of Proteus morganii (Miyaki, Hayashi, and Ando, 1958).

4-Amino-5-imidazolecarboxamide is apparently a direct precursor of purines (Brooke and Magasanik, 1954). Furthermore, paraaminobenzoic acid plays a particular role in the synthesis of purines at the stage where they are converted from 4-amino-5-imidazolecarboxamide. This position was based on the fact that 4-amino-5-imidazolecarboxamide accumulates in paraaminobenzoic acid-deficient E. coli mutants, as well as on the inhibitory effect of paraaminobenzoic acid analogs (atoxyl, paraaminoacetophenone, paraaminosalicylic acid) on purine synthesis. Paraaminobenzoic acid is apparently a coenzyme in the process of the conversion of 4-amino-5-imidazolecarboxamide to purines (Gots and Shu, 1952). Williams (1951), who used 4-amino-5-imidazolecarboxamide labeled in four carbon atoms, established that carboxamide is very rapidly incorporated into adenine and guanine by a culture of Saccharomyces cerevisiae.

When paraaminobenzoic acid is blocked by sulfamides, this leads to the accumulation of the analogs of imidazolecarboxamide in bacterial cultures (Bergmann,et al., 1952; Shive,et al., 1947).

In E. coli, the synthesis of 4-amino-5-imidazolecarboxamide is inhibited by the addition of purines to the medium (Gots, 1957).

## SUMMARY

On the basis of current data in the literature, it can be regarded as established that the utilization of purine bases by microorganisms as sources of nutrients is accompanied by their decompositon through allantoin, allantoic acid, urea, and glyoxalic acid to ammonia and carbon dioxide.

The synthesis of purine bases is carried out with the utilization of $CO_2$, $NH_4$, and a number of amino acids. Aside from the synthesis of purine bases from low molecular compounds, microorganisms are capable of assimilating existing purines from the nutrient medium, but in this case the purines are not incorporated directly into the nucleic acid molecule, but have to pass through the intermediate products stage.

The precursor of purine is 4-amino-5-imidazolecarboxamide.

The principal role in the process of the coversion of 4-amino-5-imidazolecarboxamide to purines is played by para-aminobenzoic acid,which is a coenzyme in this process.

Nutrifying microorganisms can utilize the amino group of purine derivatives in energy-yielding oxidative reactions; the conversion pathways of these compounds apparently differ from the conversion pathways in cultures of heterotrophic microorganisms.

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.

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# *REVIEWS*

## "MICROBIOLOGICAL REFERENCE JOURNAL"

Reviewed by A. A. Imshenetskii

Translated from Mikrobiologiya, Vol. 30, No. 1,
pp. 181-186, January-February, 1961

Every year the number of scientific microbiology journals is continually increasing. If fifty years ago there were not more than six specializedperiodical publications, now they number more than thirty, and it is becoming increasingly difficult to follow scientific literature.

The publication of a large number of journals in foreign languages not easily accessible to the reader complicates this problem even more. Hence, the interest shown in all countries toward abstract journals, and also toward annual publications containing a survey of current literature on specific problems, is understandable. Scientists have not reconciled with the fact that they must read scientific articles in the original concerning only the questions with which they themselves are directly working, and that that "little of everything" mentioned by K. A. Timiryazev they must obtain from surveys, monographs and reference publications. Because of this the reference journal, which enables wide circles of scientists to follow information published in various countries, has become important.

Over a period of several years the Institute of Scientific Information of the Academy of Sciences, USSR, has been publishing the Reference Journal, "Biology." For microbiologists the second issue of this Journal is interesting – it contains abstracts of articles on virology, microbiology, parasitology, and immunology. In the present review we shall examine abstracts devoted to studies in the field of general and technical microbiology and published in seventeen issues of the Journal (Nos. 19-22, 1959, and Nos. 1-13, 1960). The basic aim of this review is to note those shortcomings in the work of the Reference Journal which if eliminated will make it even more useful for wide circles of microbiologists.

### Existing Departments

Articles on those microbiological fields that are of interest to us are entered under four departments.

The first of these, "General Microbiology" is published under the editorship of Z.* M. Dikanskaya, A. S. Kruviskii, I. L. Rabotnova, Ya. I. Rautenshtein, Z. N. Robisheva, and T. P. Chernik. This department consists of the following subdivisions: 1) general questions; 2) methods and techniques of investigations; 3) systematology, morphology, cytology; 4) variation and heredity; 5) filterable forms, L-forms and microorganisms of the pleuropneumonia type; 6) physiology and biochemistry; 7) growth and development of bacterial populations; 8) microorganisms of water and air; 9) action of external factors and disinfection.

The second department is devoted to antibiosis and symbiosis and has the subdivisions: 1) antibiosis and symbiosis (editor T. P. Chernik); and 2) antibiotics (editor V. A. Shorin).

The third department is "Technical Microbiology" (editors É.* M. Dikanskaya, I. L. Rabotnova, and T. P. Chernik).

The fourth department is "The Geological Activity of Microorganisms."

The divisions listed above encompass quite completely all publications of interest to the general microbiologist; however, it is difficult to agree that the "structure" the Journal has assumed under the Editorial Staff is flawless and does not have substantial weaknesses.

* As in Russian original – Publisher.

The question first arises as to why some important aspects of microbiology have found no reflection whatever in this "structure." Thus, a special subdivision devoted to articles on the microbiology of water and air ignores earth as a medium. The Reference Journal completely lacks reference to work on soil microbiology. True, in the series "Soil Science " there is a part called "Soil Biology " which includes abstracts on soil microbiology articles. However, it is incorrect to regard soil microbiology merely as an adjunct to soil science. The microbiologist should, in one issue, be able to obtain everything that has direct bearing on his field of specialization, and it is impossible for him to build a concept of ecological development without acquainting himself with the biology of soil microbes. Such a blank in a journal of abstracts is especially undesirable if we consider that in the Republic Academies affiliated with the Academy of Sciences, USSR, and in other outlying scientific research institutions, where it is difficult to obtain a series of issues of a periodical, there are especially many soil microbiology laboratories. That is why it is extremely desirable to publish abstracts on soil microbiology in this issue of the Journal.

A second aspect undeservedly neglected by the Journal is the microbiology of foods. This is completely lacking although isolated articles connected with the use of microbes in manufacture of food products or the microbiological spoilage of same can sometimes be found in the section titled "Sanitation Microbiology." It is very difficult, however, to agree with the proposition that the question of the presence of Bacillus cereus in cream (29062), or the influence of plastic on the microflora of milk (29056), or the influence of bacteria on the pigmenting substance of muscle (29063), have more connection to sanitation than to food microbiology.* It is absolutely essential for the Journal to include abstracts on everything concerning the microbiology of milk, meat, fish, vegetables, fruits, preserves, bakery products, and also of the frozen food industry.

The organization of certain divisions in a journal of abstracts should depend not only on the wish to encompass fully the various aspects of microbiology, but to some extent the establishment of divisions should be guided by a "scientific policy " applicable to the given field. In this connection, we should emphasize that in the development of contemporary microbiology unique significance has been gained by studies in the field of the physiology and biochemistry of microbes. Perhaps the time has arrived to split this subdivision in the Journal into smaller brances, devoted, for instance, to respiration and fermentation, enzymology of microbes, photo- and chemosynthesis, etc. Such a subdivision would allow not only for a better systematization of the articles, but also broaden this division. This will be discussed in more detail below. At any rate, it is difficult to comply with the fact that the editors found it possible to present, as an independent subdivision, the "filterable forms," and did not enlarge the subdivision on physiology and biochemistry. It is more logical to unify the subdivision "filterable forms" with "morphology" or "growth and development," and, on the other hand, in the section on "physiology and biochemistry," to achieve further "biochemicalization" and to include more abstracts on the biochemistry of microbes. It is hard to accept that the division of physiology and biochemistry is given almost equal attention with the division devoted to antibiotics.

Speaking of the "structure" of the Journal, we must ask to what extent the apportionment of abstracts into divisions is correctly carried out. In some cases one encounters an incorrect classification of abstracts. Thus, articles on the variability of actinomycetes sometime land in the division "Variations" (59224), and sometimes in the division on antibiotics (59285).

## Choice of Articles for Abstraction

It is quite natural that even a journal of abstracts with a large staff at its disposal cannot possibly refer to all articles appearing in all languages within a given field. Specifically on account of this, the most responsible "operation" becomes the choice of articles for abstraction. To substantiate this choice, independent of the personal interests of the editor of the subdivision, the knowledge of the given language by the reviewer, the ease or the difficulty of the text and other considerations are factors which make the problem not always an easy one. Thus, actually, the correct selection of the most important and needed articles for the Soviet reader is the most substantial job of the editorial staff. For this purpose we need an "active" selection, stemming from the current condition of this or that scientific problem, the novelty for the country of a certain microbiological process, the theoretical and methodological significance of a given experiment, etc.

After carelly acquainting oneself with the contents of the Reference Journal, one reaches the conclusion that just this part of the work is achieved least successfully. Quite often, articles which would upon the most

*The figures in parentheses correspond to numbered abstracts in the Reference Journal.

unprejudiced evaluation present undoubted interest are not abstracted at all and only their titles are given, whereas the contents of works of comparatively little value are presented in detail. Let us cite a few examples illustrating this. Very frequently physiological and biochemical experiments, which possess – especially now, with the sharp increase of interest in functional characteristics of microbes – a principal interest, are not abstracted at all and we get only the name of the article and an indication of where it is to be found. Judging from the titles alone, it becomes apparent that the absence of abstracts on these works is a great defect in the work of such an important part of the Journal as the section on physiology and biochemistry. Let us list a few such articles: "The Purification and Chemical Properties of Flagella" (54041), "Pentose Metabolism of Bacteria" (54050), "Microbiological Methods for Vitamin $B_1$ Determination " (54076), "Proof (of the Existence) of a Cycle of Tricarboxylic Acids in Serratia marcescens" (48819), "A Study of Bacterial Proteinase" (48835 and 88836), "Transformation of Steroids by Microorganisms and the Application of these Processes in Hormone Synthesis" (43807). The number of such articles could be multiplied several times, and they would have more basis for selection as abstracts than a series of articles devoted to the morphology of the L-form or to the changed morphology of the intestinal bacillus streaked from old cultures. In this connection a question may arise. Is it worthwhile to include detailed abstracts of biochemical works on the physiology and biochemistry of microbes, when there is in existence a special biochemical abstract journal? The whole development of contemporary microbiology is inseparably connected with successes in the field of the physiology of microbes, which in its turn is wholly based on the newest biochemical data. Therefore, a microbiological abstract journal should, as fully as possible, illustrate in its pages questions on the physiology and biochemistry of microorganisms. It would be wrong to send the microbiologist to the biochemical issue of the Journal. The reader is in his right to count on finding all that is important to him as a specialist in one and the same issue. Moreover, the nature of the abstracts themselves on various biochemical problems should vary somewhat, depending on whether they are intended for the microbiological or for the biochemical issue of the Journal.

Upon familiarity with the contents of the Reference Journal, there arise a number of other questions connected with the selection of articles for abstraction. Is it worthwhile to summarize in such detail articles which are published in such widely distributed journals as The Proceedings of the Academy of Sciences, USSR; Microbiology; Journal of Microbiology, Epidemiology and Immunology? Almost all scientific reasearch institutions employing microbiologists, even those in the outlying regions, have access to these journals and the scientist can easily acquaint himself with these articles in their original. In many such cases the Reference Journal could limit itself to just placing a notation. This would free space in the Journal for summarizing work published in little circulated Soviet publications and for foreign articles. The Journal does not devote enough attention to works published in Armenian, Caucasian, Lithuanian, Uzbek, and other languages of nations of the USSR. Little is taken into account of the fact that in Japan technical microbiology and the biochemistry of microbes are being intensively developed, and that it is extremely necessary for us to summarize Japanese work in detail. Little attention is given to works published in the Spanish, Portuguese, Danish, Finnish, Swedish, and Norwegian languages. Meanwhile, interesting works on various questions of general microbiology are being published in these languages.

Speaking of widening the function of the Reference Journal, we cannot but mention the necessity to devote at least short annotations to emerging references, guides, monographs, theroretical compilations and other analogous publications. In very rare cases this is done even now, but with a great number of extremely important books, not easily accessible to the wide circle of readers, nothing but the name of the monograph is presented. To know, at least in brief, the contents of monographs emerging both in Russian and in the foreign languages is sometimes more necessary than familiarity with the contents of a single article. We shall mention only a few books, the names of which were reported in the Journal. Faulker, "Microorganisms in Organic Chemistry" (91665); Stanier, Doudoroff, and Edelberg, "General Microbiology" (91672); Pelczar and Reed, "Microbiology" (91678); d'Alexandro, "Lectures on Microbiology" (91673), Frazier, "Food Microbiology" (96157); Foster, et al., "Microbiology of Milk" (96151); Enishtya, "Food Microbiology"; Bisset, "Bacteria" (53893). Including short tables of contents of these publications would seem useful, especially if we realize that these books are frequently not available in our outlying districts.

## Preparation Period of Abstracts before Publication

It is very important for every scientist to become familiar as soon as possible with the contents of articles on questions of interest to him. This can influence the direction of his investigations, make unnecessary the continuation of experiments begun and help solve a specific problem. Therefore, the speediest possible publication of abstracts is extremely desirable. Unfortunately, the time that elapses between the appearance of an article in

print and the appearance of its abstract in the pages of the Journal is too prolonged. Not to be arbitrary, let me give the following example. The first issue of the Journal for the year 1960 contained: one abstract of an article published in 1956, 12 abstracts of articles which had appeared in print in 1957, 56 abstracts of articles published in the literature in 1958, and only seven abstracts of 1959 articles. Thus, it takes 2-3 years until the abstract of an article appears in the pages of the Reference Journal. This, of course, is too long a time and it is necessary to shorten it appreciably. We cannot explain this tardiness by saying that foreign journals are late in coming in, since articles from Soviet journals are subjected to a similarly prolonged lag before abstraction. Consequently, the blame rests on the organization of the whole process of abstracting the articles and publishing the Journal. The work of the Journal should be reorganized so as to enable the specialist to read a given abstract no later than a year and a half after publication of the article.

## Nature of the Abstracts

Should the abstracts contain critical comments or should they merely convey the article's contents? The basic aim of the Journal is to familiarize the reader with the factual data contained in the article abstracted. Any evaluation of an article's contents is a great responsibility, and when given by only one person this evaluation cannot always be unprejudiced and correct. Even with a collegiate management of the Journal it is difficult to find among members of the Editorial Staff specialists on all the questions touched upon in the various articles. Therefore, we cannot but hail the tone in which the abstracts are written—it is objective in the best sense of the word.

Many of our scientific journals have sections devoted to discussions and also review sections, and hence we have every opportunity to subject to critical analysis this or that work. It does not enter into the aims of an abstracts journal to critically analyze the contents of an article, and all responsibility for factual data, accuracy of numerical material and ideas expressed rests solely with the author of the article.

## Scope of the Abstracts

It is quite natural that the size of an abstract should depend only to some extent upon the size of the article. Of most interest to the reader are the novelty of facts reported in the abstract and their scientific significance. Therefore, it is very important to establish the size of the abstract that conveys to the reader the most valuable part of a given scientific work. The scope of the abstract should to no extent be determined by the personal interests of the reviewer, nor should it depend on the size of the remuneration payable in proportion to the length of the abstract. The Journal contains many abstracts whose scope calls for no criticism, but at the same time one can point out others that are too detailed, while some abstracts are too short. We shall look at some examples. The Proceedings of the All-Union Scientific Research Institute of the Confectionery Industry (No. 12) were abstracted in great detail: 13 abstracts on three pages. As another example, the abstract of P. F. Ievlev's work, "Investigation of Some Aspects of the Biochemistry of Sherry Fermentation," (19250) takes 51 lines; i.e., it is 2-3 times longer than the average abstract. Abstract No. 18662 and some others are too lengthy. In contrast to these, quite a number of articles are treated too briefly. Thus, an article devoted to mycobacterial aspara gine metabolism is abstracted in seven lines (54033); the article, "The Metabolism of D-Xylose by Cell-Free Extracts of Penicillium chrysogenum" is abstracted in four lines (54093). The abstract on bacterial oxidation of quinone comprises eight lines (4375). Sometimes articles of great theoretical and practical importance likewise have abstracts that are too short. Thus, research devoted to the metabolism of Leuconostoc mesenteroides, which aids in producing the blood substitute dextran, is abstracted in four lines (24128). Therefore, the editors do not pay enough attention to that harmony in the length of the abstracts which should be maintained in relation to the significance of the articles.

## Quality of the Abstracts

The overwhelming majority of abstracts in the Journal are written with a knowledge of the subject and they convey a correct impression of the article's contents. If below we indicate a series of faults, inaccuracies and mistakes found in some abstracts, this is done exclusively with the aim of aiding the editorial staff in bettering the quality of the abstracts. The latter depend to the same degree on both the reviewer and the editor of the section, and it is usually difficult for the reader to determine who made the mistake.

It is especially deplorable when facts are reported incorrectly. Thus, Abstract No. 82891 states that mycobacteria lack "the amino acid of the asparagine type." It is incorrect to call amides amino acids. Reading in Abstract No. 96071 the following phrase: "the microcolonies did not differ from the original in morphology, gram stain or serology. . .," the reader would not immediately understand that they are speaking, not of the colonies, but of bacteria taken from these colonies. Abstract No. 591, very interesting since it discusses in detail the

RNA and DNA contents of Bacillus cereus cells, reports that DNA contains a 34% molar ratio of thiamine. Sometimes it is even difficult to guess which compound is being referred to, e.g., when we encounter the following: "..."upon addition to the medium of HCH-$\gamma$-isomer in the amount of 0.8 mg . . . " (675).

The abstract should contain no abbreviations which are not explained in the text unless they are in wide general use. To quote several such abbreviations: "EDTA" (91696) – not everyone knows that this is ethylenediaminetetraacetate; the numbers of abstracts 91670 through 91681 have a capital "K" beside them, the meaning of which remains unclear to the reader.

Likewise, there should not be various abbreviated symbols describing one and the same phenomenon. In abstract No. 43692 protoplasts are designated as "PP," and on the same page in other abstracts as "P." Usually, the names of foreign authors are rendered correctly in Russian transliteration, but sometimes it is unclear why both the reviewer and the editor refuse to comply with common usuage and give quite unusual transcriptions of names. Thus, the Japanese surname Yamaguchi appears in the Journal in Russian as Yamagugi (91710, 917211); the name Braun for some reason is written in Russian as Bron. .

Special attention should be paid to the correct translation of titles of articles. Sometimes they are not translated with sufficient care. Thus, the title of the article in abstract No. 96090 is translated as: "The Interaction between Caffeine, Guanine and Adenosine in Inducing Genesis of Yeasts with Insufficient Respiration." The word "genesis" should be replaced. But sometimes the translations are altogether unsuccessful. Thus, "Autotrophic Growth of Copper-Resistant Variant of Yeast" is rendered "Autotrophic Growth in Resistance to Copper of a Variant of Yeasts" (43738). The title of an article abstracted as No. 24203 reads as follows in English: "Symbiotic Variation in Trifolium ambiguum M Bieb. with Special Reference to the Nature of Resistance." This title was translated as follows: "Symbiotic Variation of Trifolium ambiguum M Bieb., Especially in the Nature of Resistance to Contamination." The name of Bonner's article, "Aspects of Mycological Research in Japan," is translated "The Course of Microbiological Research in Japan." In translating titles the use of incorrect special terms should be avoided. In abstract No. 24238 the title of the article is translated: "Bacteria of the Coli aerogenes Group as Inducers of Swelling (Vzdutie*) in Cheese." The term "Vzdutie"* is not applicable, the accepted term for the spoilage of cheese being "vspuchivanie."* Likewise a cause for misunderstanding is the following translation of a title (29055): "Dispersion of the Rods of Brucella S-19 in the Light of Periodic Examination of Milk." The title of the article abstracted as No. 54027 is likewise peculiar. "Leakage (!) of Intracellur Substances from Cells of Bacillus megaterium."

Some abstracts reflect but little the influence of the editor. Only thus can one explain the occurrence of sentences that are so unfortunate in grammar or style, and also completely obscure in meaning. For example: "...with a hot water extract of malt seedlings, containing either xylose or glucose" (43757). Further: "The cells contain a chromatin nucleus, which the author hypothetically regards as an analog of the nucleus" (29000). Or the following sentences: "Likewise studied is the influence of purines and pyrimidines on the growth and the formation of ferments" (29028); "...a small amount of gifov*"(43741); "From the filters of a culture of intestinal bacillus" (54106).

Likewise, names of instruments and apparatuses unfamiliar to our reader should be avoided, and when they are mentioned it is necessary to describe the instrument at least briefly. To how many readers does the following sentence, contained in abstract No. 48875, mean anything: "A description is given... of the Moulton apparatus and modifications of the Porton impinger."

Sometimes incorrect terminology catches our attention. In abstract No. 19156, containing information on the systemotology of the actinomycetes, the following phrase occurs: "... the developments on the edges of spores proved to be stable features." We know of no "edges" in spores and the above should have read, instead, "spore wall surface."

tent of the most important information and facts. Unfortunately, we encounter abstracts which contain detail but lack the basic, the most substantial. For instance, in the abstract of the article "Isolation of Bacteria Synthesizing Vitamin $B_{12}$" (54056), there is no indication as to which bacteria were studied or as to how much Vitamin $B_{12}$ they formed. Naturally, the value of such an abstract is not great. When the abstract discusses the stability of sarcina pigments to radiation (48910), the reader naturally is interested in the color and nature of the pigment, but of this the abstract mentions nothing.

* Transliteration of Russian – Publisher.

## Misprints

It is, of course, practically impossible to completely avoid misprints in the publication of scientific articles and abstracts. The important point is their nature, since sometimes misprints can alter completely the sense and meaning of a sentence. The number of misprints in the published abstracts is, unfortunately, great, and they contain a series of mistakes which could properly be assigned to the category "sad."

It makes an unpleasant impression when one and the same substance in one and the same abstract has varying transcription. Thus, in abstract No. 91717 we encounter "glyutathione," and then in the next line "glutathione." In abstract No. 661 we read of the splitting of cellulose to cellobiase (correct form should be cellobiose). In editing it is necessary to observe that the initials of one and the same reviewer remain unchanged. Thus, abstract No. 29089 is signed by A. A. Rasumov, while the next, No. 29090, is signed by A. S. Rasumov.

There are also misprints in the Latin names of microorganisms: Oospova Lactis (82871); Staphilococcus (43767); Baculus subtilis (33794); Actynomyces globysporus (48825); Plicchia indica (54044).

We shall indicate a series of other misprints. The ferment "ecolase" (should be enolase) (91713); the title of an abstract says "microbacteria" (43773), where it should be "mycobacteria." We find the same mistake in abstract No. 19137. One and the same strain of Aspergillus in abstract No. 96095 is named Lis 22 in one case, and Lys 22 in another. The title of an article, "The Influence of the Concentration of Derments" ... instead of "Ferments" (5157); and "Relationship to Ancinophages" (19157). Speaking of mistakes of a technical nature, we must note that sometimes one publication is mentioned twice. Thus, for Pelczar and Reed's course on microbiology we have two annotations (43723, 91678).

The editorial staff of the Microbiological Reference Journal and the Institute of Scientific Information are fulfilling a great and extremely useful task. They enable numerous Soviet microbiologists to acquaint themselves with new scientific literature, and especially make it possible for workers in outlying areas to be in touch with all the latest achievements in the microbiological field. To the Journal goes the credit for initiative in uniting the interests of microbiologists in different specializations by organizing common sections containing references in physiology; biochemistry; variation of both pathogenic and nonparthogenic microbes. The creation of such a "common platform" leading to a healthy "integration" we cannot but commend very highly, since the need for this has long been felt. Several general departments of microbiology present an equal interest to all microbiologists, regardless of their narrow specialization. Over the years of its existence the Journal has trained cadres of qualified reviewers and editors, who devote much time and effort to this exceptionally laborious and exacting work of the selection, abstraction and editing of microbiological articles.

The above review has presented a number of critical comments, indicated mistakes and deficiencies in the work, and also expressed some wishes. If this will in some degree aid in improving the work of the Reference Journal, the aims of this review will have been fulfilled.

"MICROBIAL SELECTION" by D. F. Petrov

Edited and with a preface by Corresponding Member
AN SSSR V. L. Ryzhkov. Medgiz-Moscow, 1959.
278 pages; price 9 rubles, 45 kopecks

Reviewed by K. Kozlov

Translated from Mikrobiologiya, Vol. 30, No. 1, pp. 186-188,
January-February, 1961

There have been published in recent years in our scientific literature a comparatively large number of books devoted to questions of variation in microbes (K. V. Kosikov, G. P. Kalina, V. L. Elin, S. A. Muromtsev, V. D. Timakov, et al.,etc.). All these monographs treated the theoretical aspects of the problem and gave little attention to general methods of microbial selection.

D. F. Petrov's book will undoubtedly attract the attention of microbiologists, since it "leads the reader into all the basic problems of selection and gives descriptions of the newest selective methods " (from the preface of the editor, Corresponding Member of the AN SSSR V. L. Ryzhkov).

D. F. Petrov's book "Microbial Selelction" consists of an introduction and two parts. The first part is devoted to general methods of selection and comprises five chapters: 1) history of microbial selection; 2) selection for increased stability and lowered nutritional requirements; 3) selection for increased production of substances useful to man; 4) selection with the aim of obtaining forms with higher nutritional requirements; 5) some applicable possibilities of synthetic selection in microbes.

The second part is titled "Specific Selection in Microbes" and consists of three short chapters: 6) selection in ascomycetes; 7) bacterial selection; 8) selection in viruses.

In the introduction the author presents a short history ot selection, stressing the evolutionary theory of Ch. Darwin and the opinions of I. V. Michurin on methods of selection. The greater part of this section D. F. Petrov devotes to the presentation of concepts of corpuscular or classical genetics, with an explanation of terms and definitions.

On page 15, D. F. Petrov affirms that "mutations take place under the influence of the external environment, but are not adaptations to those environmental factors that cause them." The role of natural selection in this is purely prophylactic and consists of blemishing and eliminating harmful mutations.

Mutations are the main building material of evolution and perform their function only through changes in the DNA-gene molecules and the transmission of these changes to future generations.

On page 18, D. F. Petrov states that the transmission of hereditary factors in higher plants and animals follows Mendelian laws.

Discussing the characteristics of heredity in microbes, and in particular in bacteria, the author analyzes the phenomenon of transduction. On page 25 he writes that "under controlled laboratory conditions the transmission of individual hereditary traits . . . of the donor to some characteristics of the recipient with the aid of a completely pure preparation of nucleic acid ... was first established by MacLeod and Avery in pneumococci." (Italics mine – K. K.) (The author also discusses this on pp. 197-199.)

Such a statement on Petrov's part calls forth justified misunderstanding, since according to the authors of the work themselves the DNA preparations were not completely pure, and besides, under the influence of the DNA

with an admixture of albumen it was polysaccharides (capsules),and not albumens,that were synthesized.

The action of DNA alone in transmitting hereditary traits has not been proven in transduction either, transduction being "the transfer of individual parts of donor chromosomes by bacteriophage" onto another bacterium—the recipient (ref. p. 25 in the book).

Having summarized all the works on bacterial transformation and transduction which affirm the monopolistic role of DNA in hereditary phenomena, Hershey indicated that it was easy to draw premature conclusions and that he "expressed regret concerning certain inferences drawn from his experiments" (ref. Advances in Virus Research, IV: 51, 1957).

Similar conclusions negating the monopolistic role of DNA in transmitting hereditary traits have been reached by a number of Soviet microbiologists (A. N. Zhukov-Verezhnikov, V. D. Timakov, and others). Even D. F. Petrov himself, in the journal "Questions of Philosophy" (No. 7, 1958), has written of the participation of albumen together with DNA in phenomena of transduction and transformation.

On page 24 of the Introduction, D. F. Petrov writes that even though the presence of a nucleus in bacteria has been established, the presence in the nucleus of chromosomes and of karyokinetic mitosis is a question as yet undecided, and on page 25, discussing transduction, the author, forgetting what he wrote on page 24, writes of the transfer of individual chromosome sections with certain genes obtained upon destruction of the donor bacterium by phage (ref. also p. 197).

Thus, elucidating the concepts of classical genetics, the author himself wholly supports their positions and the whole presentation of this section is given without due critical evaluation of the experimental material he brings out.

In the first chapter, "The Histroy of Microbial Selection," D. F. Petrov examines the struggle between the pleomorphists and the monomorphists—works of L. Pasteur, L. S. Tsenkovski, and other scientists on changes in microbes and their methods of selection; the quests of S. N. Winogradsky with his principle of elective enviroments. He presents a description of the "provoked or induced" method of selection (the penicillin method of the author is based on Deziss, Lederberg, and Zinder); considers that the induced method developed by him is a continuation of I. V. Michurin's induced selection method of fruit plants and microbes; considers that his method is based on the specific action of penicillin, which eliminates all actively mebatolizing cells but does not kill cells in anabiosis (in a medium lacking essential bacterial nutrients, or at low temperatures). The author gives a detailed description of this method of selection in the fourth chapter, which is devoted to the development of forms with raised nutritional requirements; he presents the data of his investigations and also those of other authors, mainly Americans.

The second chapter, devoted to the selection of forms with heightened stability and with lowered nutritional requirements is, in our opinion, the core of D. F. Petrov's monograph, since it is here that he presents his credo on the theoretical problems of evolution—specifically on the relationship between mutational and adaptive variation. Throughout the whole chapter, and in a number of other places in the monograph (pp. 15, 49-51, 52, 57, 59, 82, etc.)the author writes that adaptive or accommodative variation occurs as the result of a new medium and persists as long as this medium exists.

Upon elimination of the factor causing the variation, this adaptation disappears, i.e., it is not stable and not established in the heredity. The author elucidates concepts of genotype and phenotype adaptations. The first occurs as a result of mutation in a few living forms (mainly microbes) in adaptation to a new medium. By phenotype adaptation the author means "the incorporation of a corresponding medium by ancestors of the given form in the phylogenetic process, in the more or less distant past" (p. 50).

This last statement of D. F. Petrov is reminiscent of C. Laille's arguments against evolution in his "Fundamentals of Geology," where he wrote that the potential ancestral forms already possessed all the faculties which were later to be developed and manifested in the process of cultivation.

In other words, the author espouses a most preadaptational position.

D. F. Petrov writes that under the influence of phage, antibiotics, x-rays and ultraviolet rays what occurs is a selection of pre-existing forms (p. 53), which had occurred as the result of chance mutations.

In the same chapter, basing himself on the stand described above, the author criticizes the work of K. V.

Kosikov, and declares that the sucrose in the experiments of this latter author caused, not a creation of mutants, but rather a multiplication of already existing or previously mutated cells (with the average frequency of one mutation per 10-100 million individual cells; see pp. 75-79 and also pp. 81-82).

D. F. Petrov writes, for instance, that in the experiments of Lederberg and Cavalii-Sfortz, there did not arise, under the influence of streptomycin, forms resistant to it, but rather that these latter had already existed in the original population (p. 82). Hereditary changes adequate to the reacting factor cannot take place, since "selection, which operates on the ancestry in general, cannot predetermine the nature of hereditary change in the offspring " (p.79).

Within such a framework, mutations are, in principle, independent of the nature of the reacting factor, and are therefore accidental to the organism.

D. F. Petrov considers that because it is difficult to imagine that sucrose (the carbohydrate in the medium) causes the reconstruction of protein into new protein (the enzyme sucrase), there cannot exist any adaptational mutation (p. 79).

But just because we do not as yet know the mechanism of such a reconstruction, it does not mean that the reconstruction is impossible. D. F. Petrov himself writes of the reconstruction of capsules, antigens, etc., under the influence of DNA in phenomena of transduction and transformation. The mechanism of this change is likewise unknown to us, but in this case it does not seem to embarrass the author.

V. L. Ryzhkov, the editor of the book, says that the author is a disciple of I. V. Michurin, and develops the views of his teacher in the field of microbial selection. But in I. V. Michurin's work we would not find any of the positions upheld by his pupil.

I. V. Michurin's main contention regarding the question under discussion was an acknowledgment of change occurring under environmental influence and subject to adaptive modification.

Michurin did not ignore other forms of variation and did not regard adaptational change as the omnipresent and only form, but he did emphasize that the latter has a great and determining significance in the evolution of living organisms. The author, on the other hand, bases on the position of Morgan's school, whose primary consideration is change invoking defect and abnormality (ref. Morgan, "Experimental Basis of Evolution," 1935, pp. 25-26), i. e., mutational, harmful change. Michurin's primary consideration was change for the better—adaptive, directed change. From this arose the new form of selection characteristic of Soviet biology—a planned-cultural selection.

D. F. Petrov opposes adaptive change as the leading form of evolutionary change, forgetting that change itself is an adaptation historically evolved by natural selection in the course of evolution. That is why nature abounds in factors which react adaptively. The mechanisms of this process are as yet unclear and need to be studied, not denied from the start, as they have been by the author of the reviewed monograph.

Powerful mutagenic factors, apparently, do not exert a leading role in the occurrence of variation in natural conditions. That is why spontaneous mutations comprise a more narrow, isolated case of adaptation, likewise caused by factors in the external medium.

In the fourth chapter the author discusses some very interesting data on selection in antibiotic producers. But here D. F. Petrov takes a stand with which it is hardly possible to agree. We have in mind Petrov's negation of the utilization, in selection, of the relationship between morphological and physiological traits.

Use of the method correlating form and function has long been known in selection. It is quite another thing that this problem is only now beginning to be applied in microbiology —in general, the laws of correlation are still "dark" and little studied (Darwin) but the negation in principle of such a relationship has no theoretical foundation.

The fifth chapter, devoted to synthetic selection, i.e., hybridization, brings abundant and interesting material with detailed descriptions of working methods for obtaining new, economically useful forms.

Elucidating the question of hybridization in bacteria, the author considers that the sexual process in bacteria has already been established, and from this makes corresponding prognoses. True, the author does not present the latest works from the Pasteur Institute in Paris on this question, but still, in the first chapters of the book (pp. 24-25, etc.) he speaks of the sexual process in bacteria as of a not yet entirely resolved phenomenon; in the present section,

however, this question seems to have been already decided for the author. .What is more, D. F. Petrov even presumes the existence of a sexual process in phages (ref. pp. 265-269).

The second part, dealing with specific selection in ascomycetes (neurospora, molds, yeasts), bacteria and viruses, gives a short description of some methods of selection in these organsims: the obtaining of live vaccines; recombinations in phages.

Considering all that has been presented above, one might say that D. F. Petrov's book offers interesting material and gives a description of working methods in microbial selection which will attract the attention of microbiologists of various types working in this field.

But the theoretical stand taken by the author on a number of questions of evolutionary theory is, in our opinion, hardly applicable. True, the editor of the book, Prof. V. L. Ryzhkov, states in the preface that he "cannot take full responsibility for the views of the author" (p. 4), and it would be quite desirable that at least in the footnotes some expression be given to the Michurin school's point of view on the subject.

# WORK WITH YEAST ORGANISMS AT SCIENTIFIC RESEARCH INSTITUTIONS IN FRANCE (PARIS)

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In the course of a scientific assignment in Paris we had the opportunity to get acquainted with the work and methods of biological and microbiological experiments conducted at the Pasteur Institute, the Institute of Biological Chemistry and the Laboratory of Physiological Genetics.

This article presents a short description of the work done with yeast organisms at the above-mentioned institutes.

At the Pasteur Institute, research with yeasts is carried out only in the department headed by P. Brechot.

The basic theme of this department is the study of the chemistry and physiology of yeasts. Much attention is devoted to the study of storage conditions of yeasts, employing lyophilization. It has been shown that yeast can be subjected to lyophilization either with preliminary drying or without.

However, their keeping qualities are better in the second case than in the first.

This department has a culture collection of the industrial yeasts of France, and supplies cultures to all the industrial institutions of the country.

The most extensive research with yeast organisms is conducted at the Laboratory of Physiological Genetics, headed by B. Ephrussi.

The main problem, on which B. Ephrussi, P. Slonimski, et al., have been working for over twelve years, is the study of the genetic traits and physiology of the so-called "petites" yeast mutants. These yeasts form "dwarf" colonies resulting from their respiratory system being considerably weakened and from the fact that they lack certain respiratory enzymes—among these, cytochrome oxidase. "Petites" mutants were obtained by two methods: by growing Saccharomyces cerevisiae yeasts on media containing acriflavin, or by prolonged cultivation in the absence of oxygen.

In the usual yeast population of S. cerevisiae these mutants occur extremely infrequently. Mutants obtained under the influence of acriflavin exhibit hereditary stability. The change taking place upon several passages of cultivation under anaerobic conditions is of a phenotypic nature.

The researchers consider the formation of the "petites" variants under the action of acriflavin to be, from the genetic point of view, a cytoplasmic mutation. The small colony size is connected with damage to the cytochrome oxidase system, which is localized in the mitochondria.

When regular yeasts were crossed with those forming "dwarf" colonies, there occurred a transfer to the latter of the ability to synthesize cytochrome oxidase. This transfer was carried out with the aid of cytoplasmic particles.

At present the work with yeast organisms in this laboratory centers mainly around the question of studying yeast respiration under conditions of aerobic and anaerobic cultivation.

One of the problems of this work is proof of the existence of specific reactions common to both the induced cytochrome oxidase biosynthesis and to the hereditarily transmitted ability to synthesize this enzyme.

The experiments are conducted by two methods: 1) by comparing aerobic and anaerobic growth in the same yeasts, and 2) by comparing the aerobic growth of the regular yeasts with that of the mutant yeasts with deficient respiration.

It was shown that if the "petites" mutants were cultivated in aerobic conditions a decrease in growth rate was observed and the yeast harvest was smaller than that of the original strain. But, after a certain number of passages (e.g, 450, which corresponds to 1000 cell generations), the mutant no longer differed from the parent strain in growth rate, but only in harvest size.

Analogous data were obtained comparing regular yeasts. The decrease in yeast biomass under anaerobic conditions is explained by the increased requirements of the yeasts for vitamins, sterols and other fat-soluble growth factors.

The cultivation of yeasts under strictly anaerobic conditions was carried out in apparatuses described in the works of Stier and his colleagues.

In this apparatus the yeasts were cultivated in an atmosphere of nitrogen over a period of a few months (only traces of oxygen were detectable).

Under these conditions it is necessary to add either ergosterol or a Tween 80 preparation containing ergosterol to the medium. Also, under Slonimski's guidance, a comparative study is being made on a group of respiratory enzymes, both in the original cultures of S. cerevisiae and in their mutants. It was shown that the mutants lose some of the respiratory enzymes, namely: cytochrome a, c-cytochromeoxidase, succinic dehydrogenase, coenzyme 1 - cytochrome C reductase, α-glycerophosphate dehydrogenase. The activity of certain other enzymes is notably weakened, e.g., malic dehydrogenase →CO 1. At the same time, the cytochrome c content is raised. On the other hand, the mutants form new enzymes; malic cytochrome c-reductose (independent of coenzyme 1) and cytochrome $a_1$ (cytochrome $a_1$ is a catalytically inactive complex). As has been indicated above, cytochrome c cannot be oxidized by molecular oxygen, since the enzyme cytochrome oxidase is lacking. However, it is hypothesized that it can be oxidized by oxidizing agents such as $H_2O_2$.

Very interesting are the results of those experiments which show that the yeast S. cerevisiae forms, under aerobic conditions, the classical lactic acid dehydrogenase (enzyme O). In anaerobic conditions, however, a completely different lactic acid dehydrogenase, which was not known earlier, is formed (enzyme N). This differs from the first enzyme in certain physicochemical characteristics and in its kinetics. This enzyme is incapable of reducing cytochrome c.

Experiments of recent years have shown that the activity of the respiratory enzymes in S. cerevisiae is stimulated to a large extent by the presence in the medium of oligosaccharides. These substances were obtained by the action of sulphuric acid on amidine and D-glucose.

It was demonstrated that under anaerobic cultivation the activity in yeasts of the enzyme alcohol dehydrogenase is considerably stepped up (about 2-3 times). The pH optimum for alcohol dehydrogenase under the above-mentioned conditions shifts from 8.4 to 7.7.

In this connection the authors have voiced the opinion that the loss of some enzymes by anaerobically cultivated yeasts is the result of the increased acidity of the medium.

Lately, with the aid of the electron microscope, a detailed study of cytological characteristics of the "petites" mutants has been initiated. These studies are being conducted by the Japanese scientist J. Iotsuyanagi, who works in Ephrussi's laboratory. He has shown that the mutants possess morphologically unchanged mitochondria, but that these mitochondria have lost a number of respiratory enzymes.

He has further establisjed that yeast cells contain a large amount of ribonucleic acid, which prevents fixatives (e.g., osmic acid) from acting on all the cell elements. Even when fixation does take place, the cell structure picture is obscured by the presence of a solid mass which results as the ribonucleic particles are coagulated by the osmic acid. Therefore, the preparation technique of the ultrathin cell slices was significantly modified. By this new method the yeast cells are preliminarily acted upon by ribonuclease.

To limit the abundant formation of ribonucleic acid in yeast cells, they can be cultivated on a synthetic medium without phosphates.

The working methods are described in detail in this author's article "Étude au microscope électronique des coupes ultrafine de la levure." [Compt. rend. Acad. Sci. 248, 274 (1959)].

Aside from staining the chondriosomes inside the cells, the Japanese scientist has separated the chondriosomes out of the cell and succeeded in staining them extracellularly.

A Polish scientist, Khlopotovski, works under Slonimski. His assignment is to investigate which enzyme is blocked in the yeast mutant that requires adenine and histidine for its growth. This mutant ($Ad_3$) was isolated from a section of colonies which had formed when the red strain of yeast ($Ad_2$) was streaked on solid media. In solving this problem, extracts were made of theoriginal yeast strain $Ad_2$ and of its mutant $Ad_3$. Enzymes were isolated from the extracts, and their nature is being determined.

We should note the experiments conducted in this laboratory by H. D. Robishon-Szulmajster. She obtained numerous mutants from various microorganisms, including some from yeasts. By way of comparing the original strain with its derived mutant she studied the biosynthetic pathways of amino acids, vitamins, purines and pyrimidines. Specifically, upon detailed study of metabolic pathways of galactose in microorganisms, she proved that these reactions take the same course as the decomposition of glucose. The decomposition of glucose, however, takes place only after the phosphorylation of galactose and the union of the latter with nucleotides.

She showed also that many microorganisms are capable of synthesizing the vitamin mesoinositol, which is essential in pyrimidine metabolism.

At the Institute of Physicochemical Biology, work with yeasts is done in the Department of Biochemistry, headed by M. Grunberg-Manago.

This department deals mainly with studying the fermentative synthesis of ribonucleotides. They study the interdependence between protein synthesis and nucleic acid synthesis in yeasts and bacteria. From various bacteria, an enzyme has been isolated which catalyzes the synthesis of ribonucleotides from nucleoside-5-diphosphat

At present another enzyme which catalyzes the synthesis of deoxypolynucleotides from deoxy-5-triphosphates has also been isolated. Another enzyme system which also catalyzes the synthesis of polynucleotides has been isolated from yeasts, but it differs from the analogous bacterial systems. This enzyme was name polynucleotidephosphorylase. It was shown that the polynucleotide formed consists not only of nucleoside-monophosphate units, but contains excess phosphate groupings. Grunberg-Manago feels that these experiments support Brachet's hypothesis of the possible existence of a nucleic acid phosphate. Phosphorylated nucleic acid probably serves as a phosphorylating agent, and during the synthesis of proteins determines their specificity. This phosphorylating agent influences the distribution of amino acids in the polynucleotide chain. [Investigations in this direction were published in the Russian journal "Biokhimiya" 23, No. 2 (1958)].

Co-worker Tavlizkii is studying the composition of ribonucleic acids in the regular S. cerevisiae yeasts and in the "petites" mutants developed by Slonimski. The results obtained from these experiments are interesting to the whole field of biology.

The experiments of Ephrussi, Slonimski, and their colleagues are greatly increasing our knowledge of the general mechanisms of variation and heredity in different organisms.

Hereditarily modified yeasts with a reduced cytochrome oxidase system and a weakened respiratory process can be obtained by various means. Russian scientists have also done some work in this direction, and have demonstrated that mutants occur under the influence of radiation energy, chemical substances (Meisél; Gauze) and the temperature factor (Meisel; Loginova).

In conclusion we shall briefly mention some industrial institutions in France which engage in the production of fodder yeasts. These institutions are intersting in that they employ a simplified and rational technological scheme in the production of fodder yeasts.

For obtaining fodder yeasts, aside from using slops (which is the waste product of alcohol plants utilizing hydrolyzatesand sulfite liquor), lactose whey can also be utilized as a medium. All these innovations in the technical scheme of the above-mentioned institutions are carried out under the direct supervision of engineer-inventor. Le France.

During the pre-factory stages the yeasts are accumulated in the Pasteur Institute's Yeast Laboratory of Prof. Brechot. On lactose whey medium the yeasts are grown by the method of continuous cultivation. When the fermentation process is ended, all the liquid is concentrated and it is then dried between two rollers in a specially constructed oven at 130-150 C. The dried mass is cream colored, has a slightly acid taste (due to the presence of lactose whey), and has a pleasant odor. The dried sheets are ground in a mill. The flour is packed into cello-

phane bags, which are tightly sealed. This plant is completely automated; therefore, during the evening and night shifts many processes go on without the supervision of workers.

As we examined the diagramatic scheme for cultivating fodder yeast on the slops of sulfite liquors, we found this method to be somewhat more involved, since it requires washing and separating of the yeasts. The yeasts are then enriched with vitamins $B_1$ and $B_2$, and irradiated for enrichment with the D vitamins.

# ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

| Abbreviation (Transliterated) | Significance |
|---|---|
| AMN SSSR | Academy of Medical Sciences, USSR |
| AN SSSR | Academy of Sciences, USSR |
| BIN | Biological Institute, Botanical Institute |
| FTI | Institute of Physiotherapy |
| GONTI | State United Sci-Tech Press |
| GOST | All Union State Standard |
| GRRRI | State Roentgenology, Radiology, and Cancer Institute |
| GTTI | State Technical and Theoretical Literature Press |
| GU | State University |
| I Kh N | Scientific Research Institute of Surgical Neuropathology |
| IL (IIL) | Foreign Literature Press |
| IONKh | Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov) |
| IP | Soil Science Inst. (Acad. Sci. USSR) |
| ISN (Izd. Sov. Nauk) | Soviet Science Press |
| Izd. | Press |
| LEM | Laboratory for experimental morphogenesis |
| LENDVI | Leningrad Inst. of Dermatology and Venereology |
| LEO | Laboratory of Experimental Zoology |
| LIKhT | Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases |
| LIPZ | Leningrad Inst. for Study of Occupational Diseases |
| LIPK | Leningrad Blood Transfusion Institute |
| Medgiz | State Medical Literature Press |
| MOPISh | Moscow Society of Apiculture and Sericulture |
| MVI | Moscow Veterinary Institute |
| MZdrav | Ministry of Health |
| MZI | Moscow Zootechnical Institute |
| LOKhO | Leningrad Society of Orthopedic Surgeons |
| NIIZ | Scientific Research Institute of Zoology |
| NINKhI | Scientific Research Institute of Neurosurgery |
| NIU | Scientific Institute for Fertilizers |
| NIUIF | Scientific Research Institute of Fertilizers and Insecticides |
| NIVI | Veterinary Scientific Research Institute |
| ONTI | United Sci.-Tech. Press |
| OTI | Division of Technical Information |
| RBO | Russian Botanical Society |
| ROP | Russian Society of Pathologists |
| SANIIRI | Central Asia Scientific Research Institute of Irrigation |
| SANIISh | Central Asia Scientific Research Institute of Sericulture |
| TsNII | All-Union Central Scientific Research Institute |
| TsNTL | Central Scientific and Technical Laboratory |
| VASKhNIL | All-Union Academy of Agricultural Sciences |
| VIG | All-Union Institute of Helminthology |
| VIEM | All-Union Institute of Experimental Medicine |
| VIR | All-Union Institute of Plant Cultivation |
| VIUAA | All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering |
| VIZR | All-Union Institute of Medical and Pharmaceutical Herbs |
| VNIRO | All-Union Scientific Institute of Fishing and Oceanography |
| ZIN | Zoological Inst. (Acad. Sci. USSR) |

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

# Soviet Journals Available in Cover-to-Cover Translation

| ABBREVIATION | RUSSIAN TITLE | TITLE OF TRANSLATION | PUBLISHER | TRANSLATION BEGAN Vol. | Issue | Year |
|---|---|---|---|---|---|---|
| AÉ | Atomnaya énergiya | Soviet Journal of Atomic Energy | Consultants Bureau | 1 | 1 | 1956 |
| Akust. zh. | Akusticheskii zhurnal | Soviet Physics – Acoustics | American Institute of Physics | 1 | 1 | 1955 |
| | Antibiotiki | Antibiotics | Consultants Bureau | 4 | 1 | 1959 |
| Astr(on). zh(urn). | Astronomicheskii zhurnal | Soviet Astronomy–AJ | American Institute of Physics | 34 | 1 | 1957 |
| Avto(mat). svarka | Avtomaticheskaya svarka | Automatic Welding | British Welding Research Association (London) | | 1 | 1959 |
| | Avtomatika i Telemekhanika | Automation and Remote Control | Instrument Society of America | 27 | 1 | 1956 |
| | Biofizika | Biophysics | National Institutes of Health* | | 1 | 1957 |
| | Biokhimiya | Biochemistry | Consultants Bureau | 21 | 1 | 1956 |
| Byull. éksp(erim). biol. i med. | Byulleten' éksperimental'noi biologii i meditsiny | Bulletin of Experimental Biology and Medicine | Consultants Bureau | 41 | 1 | 1959 |
| DAN (SSSR) / Dokl(ady) AN SSSR | Doklady Akademii Nauk SSSR | The translation of this journal is published in sections, as follows: | | | | |
| | Life Sciences | Doklady Biochemistry Section | American Institute of Biological Sciences | 106 | 1 | 1956 |
| | Life Sciences | Doklady Biological Sciences Sections (Includes: Anatomy, biophysics, cytology, ecology, embryology, endocrinology, evolutionary morphology, genetics, histology, hydrobiology microbiology, morphology, parasitology, physiology, zoology sections) | American Institute of Biological Sciences | 112 | 1 | 1957 |
| | Life Sciences | Doklady Botanical Sciences Sections (Includes: Botany, phytopathology, plant anatomy, plant ecology, plant embryology, plant physiology, plant morphology sections) | American Institute of Biological Sciences | 112 | 1 | 1957 |
| | Chemical Sciences | Proceedings of the Academy of Sciences of the USSR, Section: Chemical Technology | Consultants Bureau | 106 | 1 | 1956 |
| | Chemical Sciences | Proceedings of the Academy of Sciences of the USSR, Section: Chemistry | Consultants Bureau | 106 | 1 | 1956 |
| | Chemical Sciences | Proceedings of the Academy of Sciences of the USSR, Section: Physical Chemistry | Consultants Bureau | 112 | 1 | 1957 |
| | Earth Sciences | Doklady Earth Sciences Sections (Includes: Geochemistry, geology, geophysics, hydrogeology, mineralogy, paleontology, petrography, permafrost sections) | American Geological Institute | 124 | 1 | 1959 |
| | Earth Sciences | Proceedings of the Academy of Sciences of the USSR, Section: Geochemistry | Consultants Bureau | 106-123 | 1-6 | 1957-1958 |
| | Earth Sciences | Proceedings of the Academy of Sciences of the USSR, Sections: Geology | Consultants Bureau | 106-123 | 1-6 | 1957-1958 |
| | Mathematics | Doklady Soviet Mathematics | The American Mathematics Society | 131 | 1 | 1961 |
| | Physics | Soviet Physics–Doklady (Includes: Aerodynamics, astronomy, crystallography, cybernetics and control theory, electrical engineering, energetics, fluid mechanics, heat engineering, hydraulics, mathematical physics, mechanics, physics, technical physics, theory of elasticity sections) | American Institute of Physics | 106 | 1 | 1956 |
| | Physics | Proceedings of the Academy of Sciences of the USSR, Applied Physics Sections (does not include mathematical physics or physics sections) | Consultants Bureau | 106-117 | 1 | 1956-1957 |
| Derevoobrabat. prom-st'. | Derevoobrabatyvayushchaya promyshlennost' | Wood Processing Industry | Timber Development Association (London) | | 9 | 1959 |
| | Élecktrosvyaz | Telecommunications | Massachusetts Institute of Technology* | | 1 | 1957 |
| Éntom(ol). oboz(renie) | Éntomologicheskoe obozrenie | Entomological Review | American Institute of Biological Sciences | 38 | 1 | 1959 |
| Farmakol. (i) toksikol(ogiya) | Farmakologiya i toksikologiya | Pharmacology and Toxicology | Consultants Bureau | 20 | 1 | 1957 |
| FMM | Fizika metallov i metallovedenie | Physics of Metals and Metallography | Acta Metallurgica* | 5 | 1 | 1957 |
| Fiziol. zhurn. SSSR (im. Sechenova) | Fiziologicheskii zhurnal im. I. M. Sechenova | Sechenov Physiological Journal USSR | National Institutes of Health* | | 1 | 1957 |
| Fiziol(ogiya) rast. | Fiziologiya rastenii | Plant Physiology | American Institute of Biological Sciences | 4 | 1 | 1957 |
| | Geokhimiya | Geochemistry | The Geochemical Society | | 1 | 1958 |
| FTT | Fizika tverdogo tela | Soviet Physics–Solid State | American Institute of Physics | 1 | 1 | 1959 |
| Izmerit. tekh(nika) | Izmeritel'naya tekhnika | Measurement Techniques | Instrument Society of America | | 1 | 1959 |
| Izv. AN SSSR, O(td). Kh(im). N(auk) | Izvestiya Akademii Nauk SSSR: Otdelenie khimicheskikh nauk | Bulletin of the Academy of Sciences of the USSR: Division of Chemical Sciences | Consultants Bureau | | 1 | 1952 |

*continued*

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| Izv. AN SSSR, O(td). T(ekhn). N(auk): Met(all). i top. | (see Met. i top.) | | | | | |
| Izv. AN SSSR Ser. fiz(ich). | Izvestiya Akademii Nauk SSSR: Seriya fizicheskaya | Bulletin of the Academy of Sciences of the USSR: Physical Series | Columbia Technical Translations | | 1 | 1954 |
| Izv. AN SSSR Ser. geofiz. | Izvestiya Akademii Nauk SSSR: Seriya geofizicheskaya | Bulletin (Izvestiya) of the Academy of Sciences USSR: Geophysics Series | American Geophysical Union | | 1 | 1954 |
| Izv. AN SSSR Ser. geol. | Izvestiya Akademii Nauk SSSR: Seriya geologicheskaya | Izvestiya of the Academy of Sciences of the USSR: Geologic Series | American Geological Institute | | 1 | 1958 |
| Kauch. i rez. | Kauchuk i rezina | Soviet Rubber Technology | Research Association of British Rubber Manufacturers | 18 | 1 | 1959 |
| | Kinetika i kataliz | Kinetics and Catalysis | Consultants Bureau | 1 | 3 | 1960 |
| | Koks i khimiya | Coke and Chemistry USSR | Coal Tar Research Association (Leeds, England) | | 1 | 1958 |
| Kolloidn. zh(urn). | Kolloidnyi zhurnal | Colloid Journal | Consultants Bureau | 14 | 1 | 1952 |
| | Kristallografiya | Soviet Physics – Crystallography | American Institute of Physics | 2 | 1 | 1957 |
| Metalov. i term. obrabot. metal. | Metallovedenie i termicheskaya obrabotka metallov | Metal Science and Heat Treatment of Metals | Acta Metallurgica | 6 | 1 | 1958 |
| | Metallurg | Metallurgist | Acta Metallurgica | | 1 | 1957 |
| Met. i top. | Metallurgiya i topliva | Russian Metallurgy and Fuels | Eagle Technical Publications | | 1 | 1960 |
| Mikrobiol. | Mikrobiologiya | Microbiology | American Institute of Biological Sciences | 26 | 1 | 1957 |
| OS | Optika i spektroskopiya | Optics and Spectroscopy | American Institute of Physics | | 6 | 1959 |
| | Pochvovedenie | Soviet Soil Science | American Institute of Biological Sciences | | 1 | 1958 |
| | Priborostroenie | Instrument Construction | British Scientific Instrument Research Association | | 1 | 1959 |
| Pribory i tekhn. éks(perimenta) | Pribory i tekhnika éksperimenta | Instruments and Experimental Techniques | Instrument Society of America | | 1 | 1957 |
| Prikl. matem. i mekh. | Prikladnaya matematika i mekhanika | Applied Mathematics and Mechanics | American Society of Mechanical Engineers | | 1 | 1958 |
| PTÉ | (see Pribory i tekhn. éks.) | | National Research Council of Canada | | | |
| | Problemy Severa | Problems of the North | Massachusetts Institute of Technology* | 12 | 1 | 1957 |
| Radiotekh. | Radiotekhnika | Radio Engineering | Massachusetts Institute of Technology* | 2 | 1 | 1957 |
| Radiotekh. i élektronika | Radiotekhnika i élektronika | Radio Engineering and Electronics | Production Engineering Research Assoc. | | 1 | 1959 |
| | Stanki i instrument | Machines and Tooling | Iron and Steel Institute | | 1 | 1959 |
| | Stal' | Stal (In English) | Consultants Bureau | 13 | 1 | 1956 |
| Stek. i keram. | Steklo i keramika | Glass and Ceramics | British Welding Research Association | | 4 | 1959 |
| Svaroch. proiz-vo | Svarochnoe proizvodstvo | Welding Production | Society for Industrial and Applied Mathematics | | 1 | 1956 |
| Teor. veroyat. i prim. | Teoriya veroyatnostei i ee primenenie | Theory of Probability and Its Applications | Primary Sources | | 1 | 1960 |
| Tsvet. Metally | Tsvetnye metally | Nonferrous Metals | American Institute of Physics | 66 | 1 | 1958 |
| UFN | Uspekhi fizicheskikh Nauk | Soviet Physics – Uspekhi (partial translation) | The Chemical Society (London) | | 1 | 1960 |
| UKh | Uspekhi khimii | Russian Chemical Reviews | London Mathematical Society | 15 | 1 | 1960 |
| UMN | Uspekhi matematicheskikh nauk | Russian Mathematical Surveys | | | | |
| Usp. fiz. nauk | (see UFN) | | | | | |
| Usp. khim(ii) | (see UKh) | | | | | |
| Usp. matem. nauk | (see UMN) | | | | | |
| Usp. sovr. biol. | Uspekhi sovremennoi biologii | Russian Review of Biology | Oliver and Boyd | | 48 | 1959 |
| Vest. mashinostroeniya | Vestnik mashinostroeniya | Russian Engineering Journal | Production Engineering Research Assoc. | | 4 | 1959 |
| Vop. gem. i per. krovi | Voprosy gematologii i perelivaniya krovi | Problems of Hematology and Blood Transfusion | National Institutes of Health* | | 1 | 1957 |
| Vop. onk. | Voprosy onkologii | Problems of Oncology | National Institutes of Health* | | 1 | 1957 |
| Vop. virusol. | Voprosy virusologii | Problems of Virology | National Institutes of Health* | | 1 | 1957 |
| Zav(odsk). lab(oratoriya) | Zavodskaya laboratoriya | Industrial Laboratory | Instrument Society of America | 25 | 1 | 1959 |
| ZhAKh Zh. anal(it). khimii | Zhurnal analiticheskoi khimii | Journal of Analytical Chemistry USSR | Consultants Bureau | 7 | 1 | 1952 |
| ZhETF<br>Zh. éksperim. i teor. fiz. | Zhurnal éksperimental'noi i theoreticheskoi fiziki | Soviet Physics–JETP | American Institute of Physics | 28 | 1 | 1955 |
| ZhFKh Zh. fiz. khimii | Zhurnal fizicheskoi khimii | Russian Journal of Physical Chemistry | The Chemical Society (London) | | 7 | 1959 |
| ZhMÉI Zh(urn). mikrobiol. épidemiol. i immunobiol. | Zhurnal mikrobiologii, épidemiologii i immunobiologii | Journal of Microbiology, Epidemiology and Immunobiology | National Institutes of Health* | | 1 | 1957 |
| ZhNKh<br>Zh(urn). neorgan(ich). khim(ii) | Zhurnal neorganicheskoi khimii | The Russian Journal of Inorganic Chemistry | The Chemical Society (London) | | 1 | 1959 |
| ZhOKh<br>Zh(urn). obshch(ei) khimii | Zhurnal obshchei khimii | Journal of General Chemistry USSR | Consultants Bureau | 19 | 1 | 1949 |
| ZhPKh<br>Zh(urn). prikl. khimii | Zhurnal prikladnoi khimli | Journal of Applied Chemistry USSR | Consultants Bureau | 23 | 1 | 1950 |
| ZhSKh<br>Zh(urn). strukt. khimii | Zhurnal strukturnoi khimii | Journal of Structural Chemistry | Consultants Bureau | 1 | 1 | 1960 |
| ZhTF<br>Zh(urn). tekhn. fiz. | Zhurnal teknicheskoi fiziki | Soviet Physics–Technical Physics | American Institute of Physics | 26 | 1 | 1956 |
| Zh(urn). vyssh. nervn. deyat. (im. Pavlova) | Zhurnal vysshei nervnoi deyatel'nosti (im. I. P. Pavlova) | Pavlov Journal of Higher Nervous Activity | National Institutes of Health* | | 1 | 1958 |

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**Arachnida.** Vol. IV, No. 2. Fauna of the U.S.S.R. *By B. I. Pomerantzev.*
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**Arachnoidea.** Vol. VI, No. 1. Fauna of the U.S.S.R. *By A. A. Zachvatkin.*
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This is a translation of all papers in the Biological Sciences Sections of the Proceedings of the Academy of Sciences of the USSR. It includes sections in many areas of biology, among which are the following: biophysics, cytology, embryology, experimental morphology, genetics, histology, hydrobiology, microbiology, morphology, physiology, and zoology.

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